NONO and its Homologs Regulate the Circadian Clock

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Diese Dissertation ist meinen Eltern gewidmet.

Meiner Mutter,
die mich gelehrt hat mein Leben mit Hingabe zu leben und zu Lernen um des Verstehens willen,

und meinem Vater,
der mich gelehrt hat fortwährend die Dinge in Frage zu stellen und für die Sache, die mir am Herzen liegt, einzustehen.
(Möge er in Frieden ruhen.)

"Niczego w życiu nie należy się obawiać, tylko próbować zrozumieć. Teraz jest czas, aby zrozumieć więcej, więc możemy mniej się obawiać."

Maria Skłodowska-Curie (1867 - 1934)

This thesis is dedicated to my parents.

My Mother,
who taught me to live with dedication and to learn for the pleasure of understanding,

and my Father,
who taught me to question continuously and stand up for the things I love.
(May he rest in peace)

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Maria Skłodowska-Curie (1867 - 1934)

TABLE OF CONTENTS

Abbreviations and Acronyms

ADF	Adult dermal fibroblast
ARNTL	Aryl hydrocarbon receptor nuclear translocator
bHLH	basic helix-loop-helix
BMAL1	Brain-muscle-arnt-like-protein 1 (= ARTNL)
cAMP	3,5-cyclic adenosine monophosphate
CK1	Casein kinase 1
CREB	cyclic AMP response element binding protein
CRY	Cryptochrome
CT	circadian time
DD	Dark/Dark (constant darkness)
DBP	albumin D site-binding protein
GABA	γ-aminobutyric acid
LD	Light/Dark
LL	Light/Light (constant light)
NONO	Non-A POU octamer binding
NOPS	NONO and PSPC domain
PAS	per-arnt-sim (protein domain)
PER	Period
Rev-Erbα	nuclear orphan receptor protein family member, encoded by *NR1D1*
SCN	suprachiasmatic nucleus
TIM	Timeless
WDR5	WD repeat-containing protein 5
ZT	Zeitgeber time

Summary

The circadian clock is an endogenous molecular oscillator based on simple transcriptional, translational, and post-translational feedback loops. Therefore it can be found in such unicellular organisms as cyanobacteria and acetabularia. In mammals there is a strict hierarchy with a master clock residing in the suprachiasmatic nuclei (SCN) and peripheral oscillators in almost all body parts.

The circadian clock in mammals is manifested in daily rhythms of physiological and behavioral outputs that have a cycle length of approximate a day, i.e. sleep/wake cycles, diurnal heartbeat rates and hormone levels in blood. Circadian rhythms of behavior manifest themselves only after birth. Nevertheless, perhaps since circadian clocks are based on cell-autonomous mechanisms, functional clocks can be already observed in mouse embryonic fibroblasts. The first study presented here demonstrated no functional circadian clock in embryonic stem (ES) cells, possibly resulting from misregulation of core clock components – downregulation of the circadian transcriptional activator *Bmal1* and the repressor *Per2* as well as the overexpression of the repressor *Cry1*. After differentiating these ES cells into neurons, circadian oscillations were observed in gene expression. Similar oscillations were seen in a neural precursor cell (NPC) line, one of the earliest cell types observed during embryonic maturation. We could therefore conclude that at a molecular level, the circadian clock begins at the very earliest stages of mammalian development.

In mammals the circadian clock consists of several core components: the transcriptional activators BMAL1 and CLOCK heterodimerize and bind to E-box promoter elements of the repressor genes Periods (*Per1*, *Per2*) and Cryptochromes (*Cry1*, *Cry2*). They form a circadian repressor complex and shuttle back to the nucleus to repress their own transcription and that of clock output genes. In this repressor complex, various additional proteins were found to be associated which execute coregulatory functions. One of them was non-POU domain containing, octamer-binding protein (NONO), a protein involved in diverse nuclear processes ranging from transcription and splicing to mRNA shuttling and retention.

During the second project of this thesis, to investigate NONO function within the circadian clock and its mechanism of action, *Nono*-deficient ($Nono^{gt}$) mice were produced. Adult dermal fibroblasts (ADFs) from these mice were found to hyperproliferate due to reduced cellular senescence. NONO was found to bind in circadian fashion the promoter of *p16-Ink4a*, a retinoblastoma pathway regulator and cell cycle check point. $Nono^{gt}$ mice lacking this binding also lost the circadian expression pattern of *p16-Ink4a*. In addition to reduced senescence, a doubling in the S phase cell population was found in $Nono^{gt}$ mice.

The study of cell cycle stage distribution during the 24-hour day revealed that whereas the S phase of wildtype ADFs was gated, *Nono*^{gt} cells lost this regulation. Furthermore, lack of either NONO or essential core clock components eliminated cell cycle gating *in vitro*, and resulted in defective wound healing *in vivo*.

Despite the previous finding that *NonA*-deficient flies show arrhythmic behavior, *Nono*^{gt} mice had a moderate period shortening of 20 minutes. Investigation of conserved domain architecture revealed two close homologs of NONO, namely splicing factor proline/glutamine-rich protein (SFPQ) and paraspeckle component 1 protein (PSPC1). All three of them share tandem RNA-biding motifs and a “NOPS” domain, an extended basic Helix-Loop-Helix (bHLH) motif. Previously they have been attributed to the family of DBHS (Drosophila behavior, human splicing) proteins, which are members of nuclear paraspeckles and share such nuclear functions as splicing and mRNA retention. The third study characterized the involvement of DBHS/NOPS proteins in the circadian clock. In bioluminescence assays, deregulated levels of either of these homologs resulted in abrogation of circadian rhythms. NONO was also found to bind in circadian fashion the promoter sites of *Rev-Erbα* and *Dpb*, thereby confirming its role as a transcriptional coregulator within the circadian clock. This function was shared by SFPQ but not by PSPC1: the former was found to bind in circadian fashion to clock gene promoters and interact with both NONO and PER1/PER2. In vivo, SFPQ-deficient mice (*Sfpq*^{gt}) show shortening of period length, but those lacking the third paraspeckle factor PSPC1 (*PSPC1*^{gt}) or cells lacking paraspeckles themselves have normal clock function.

The complete work presented here demonstrates that RNA/DNA-binding proteins of the DBHS/NOPS family are involved in the transcription regulatory mechanisms of the circadian clockwork.

I. Zusammenfassung (German Summary)

Die zirkadiane Uhr ist ein endogener molekularer Oszillator, der aus transkriptionellen, translationellen und posttranslationellen Rückkopplungsschleifen aufgebaut ist. Da diese Mechanismen in Einzellern wie Cyanobakterien und Acetabularia wirken, besitzen diese Organismen auch eine zirkadiane Uhr. Im Säugetier besteht eine strikte Hierarchie zwischen der Hauptuhr in den suprachiasmatischen Kernen (SCN) in der Hirnbasis und den peripheren zirkadianen Uhren, die in fast allen verschiedenen Zellen des Körpers vorkommen.

Die zirkadiane Uhr im Säugetier lässt sich anhand von tagtäglichen Rhythmen in Physiologie und Verhalten beobachten, die einen Zyklus von etwa einer Tageslänge haben, wie z.B. Schlaf- und Ruhephasen, Herzschlagrate sowie der schwankende Hormonspiegel im Blut. Zirkadiane Verhaltensweisen sind erst nach der Geburt ersichtlich. Trotzdem kann man zirkadiane Rhythmen bereits in embryonalen Fibroblasten beobachten, vielleicht weil sie auf zellautonomen Mechanismen wie Transkription und Translation beruhen. Die erste hier vorgelegte Arbeit demonstriert, dass in embryonalen Stammzellen (ES Zellen) keine funktionierende zirkadiane Uhr am Werk ist. Dies ist wahrscheinlich auf die transkriptionelle Fehlregulation von zentralen Komponenten der zirkadianen Uhr zurückzuführen – Herabregulierung des zirkadianen Transkriptionsaktivators *Bmal1* und des Repressors *Per2* sowie der Hochregulierung des Repressors *Cry1*. Nachdem embryonalen Stammzellen in Neuronen ausdifferenziert wurden, waren typische tägliche Schwankungen in den zentralen Komponten der zirkadianen Uhr zu beobachten. Diese zirkadianen Wechsel in Transkriptionsleveln waren auch in neuronalen Vorgängerzellen (NPC) zu sehen, bei welchen es sich um eine der frühsten Zelltypen handelt, der von embryonalen Stamzellen abstammt. Daraus die Schlussfolgerung, dass die zirkadiane Uhr bereits im frühesten Stadium der Säugetierentwicklung auf der molekularen Ebene funktionstüchtig ist.

In Säugetieren besteht die zirkadiane Uhr aus verschiedenen zentralen Komponenten: den Transkriptionsaktivatoren BMAL1 und CLOCK, welche einen Komplex bilden und sich an E-Box Motiven in Genpromoteren der Transkriptionsrepressoren Periods (*Per1*, *Per2*) sowie Cryptochromes (*Cry1*, *Cry2*) anlagern. Diese Repressorproteine bilden im Zellplasma den zirkadianen Repremierungskomplex und kehren zurück in den Zellkern, um ihre eigene Transkription sowie die der zirkadian-kontrollierten Gene zu unterdrücken. In diesem zirkadianen Repremierungskomplex wurden zahlreiche zusätzliche Proteine gefunden, die eine Auswirkung auf seine Aktivität und Lokalisierung haben. Eines dieser neuen Proteine ist das non-POU domain containing, octamer-binding Protein (NONO), welches bereits zuvor in diversen anderen zellkernspezifischen Prozessen, wie

Transkription und Spleissen sowie Boten-RNA Beförderung und Rückhaltung, beschrieben wurde.

In der zweiten hier vorgelegten Arbeit wurden Mäuse denen NONO fehlt (*Nono*^{gt}) generiert, um die Funktion und die Wirkungsweise von NONO in der zirkadianen Uhr zu studieren. Die adulten dermalen Fibroblasten (ADFs) dieser Mäuse wiesen ein erhöhtes Zellwachstum auf, das sich auf eine Reduzierung der Zellalterung (Seneszenz) zurückführen liess. Es wurde gezeigt, dass NONO in täglich fluktuierender (zirkadian) Art und Weise an den Promoter des *p16-Ink4a* Gens bindet, welches ein wichtiger Zellzykluskontrollpunkt ist und zudem den Retinoblastoma Signalweg reguliert. In *Nono*^{gt} Mäusen fehlte diese zirkadiane Genexpression von *p16-Ink4a*, da NONO am selbigen Promoter nicht präsent war. Zusätzlich zur reduzierten Zellalterung, wurde eine Verdopplung der Zellen in der S Phase des Zellzyklus in *Nono*^{gt} Mäusen festgestellt. In einer 24-Stunden Zellzyklusstudie mit ADFs konnte der tägliche Anstieg an Zellen in der S Phase in einem spezifischen Zeitfenster (sogenanntes Zellzyklus Gating) beobachtet werden, wohingegen die ADFs der *Nono*^{gt} Mäuse diesen Kontrollmechanismus verloren hatten. Zudem war das Zellzyklus Gating auch in ADFs von Mäusen mit defekten zentralen Komponenten der zirkadianen Uhr nicht mehr vorhanden. Dies führte *in vivo* zu gestörter und unzureichender Wundheilung.

In *Nono*^{gt} Mäusen wurde im zirkadianen Verhalten eine Periodenverkürzung von lediglich 20 Minuten festgestellt, obwohl Fliegen mit einem defekten *NonA* Gen, dem Homolog von NONO, arrhythmisches Verhalten aufweisen. Durch Analyse der Architektur von konservierten Proteindomänen konnten zwei nahe Verwandte von NONO gefunden werden, dies waren Spleissfaktor Prolin/Glutamin-reiches Protein (SFPQ) und Paraspeckle Komponente 1 Protein (PSPC1). Diese drei Proteine besitzen alle zwei aufeinanderfolgende RNA-Bindemotiv und einer “NOPS“ Domäne, welche eine erweitertes basisches Helix-Loop-Helix (bHLH) Motiv darstellt. Im Vorfeld, wurden diese drei Proteine bereits der Familie der DBHS (Drosophila behavior, human splicing) Proteine zugeschrieben, da sie alle Mitglieder der nuklearen Paraspeckles sind und gemeinsame Zellkernfunktionen wie Spleissen und Boten-RNA Rückhaltung besitzen. Die dritte hier vorgelegte Studie, befasst sich mit der Beteiligung der drei DBHS/NOPS Proteine am Mechanismus der zirkadianen Uhr. In Biolumineszenz Versuchen konnte gezeigt werden, dass eine geänderte Genexpression von jedem der drei Homologe sich in einer fehlerhaften zirkadianen Oszillation bemerkbar machte. Es wurde gezeigt, dass NONO auch die Promoteren von *Rev-Erbα* und *Dpb* auf eine zirkadiane Art und Weise bindet. Dies bekräftigt seine Rolle als transkriptioneller Koregulator in der zirkadianen Uhr. Das Binden an zirkadian regulierte Promoteren konnte auch bei SFPQ beobachtet, PSPC1 war jedoch nicht präsent. SFPQ konnte zudem mit NONO sowie den zirkadianen

Repressorproteinen PER1/PER2 direkte Bindungen eingehen. In Mäusen denen SFPQ fehlte (*Sfpq*^{gt}), wurde im zirkadianen Verhalten eine Periodenverkürzung beobachtet, wohingegen Mäuse ohne PSPC1 (*PSPC1*^{gt}) oder funktionelle Paraspeckles keine Abnormalitäten in der zirkadianen Uhr aufwiesen.

Die hier vorgelegte Gesamtstudie demonstriert, dass RNA/DNA-bindende Proteine der DBHS/NOPS Familie in den traskriptionellen Regulationsmechanismen der zirkadianen Uhr involviert sind.

Chapter 1 - Introduction

Circadian Rhythms – Nature's Clock

Part of this section (Chapter 1.5) has previously been published as a review:
Kowalska E. and Brown S.A.: Peripheral Clocks - Keeping up with the Master Clock.
CSH Symposium LXXII 2007, p. 301-307

Basics to Chronobiology

The introduction is built up for non-chronobiologists. First, we will set the stage and have a look at how circadian clocks evolved in different organisms, and at the beginnings of circadian research in different model organisms. Thereafter we will introduce the building blocks of a general oscillator and the principles of gene oscillators. Following will be the molecular basis of a circadian clock from bacteria to mammals and how these oscillators are fine-tuned to a 24-hour day. Next will be the interplay between the master clock and the peripheral clocks. And last, the chapter about physiology and cellular mechanisms will show the importance of this particular gene oscillator in real life and its impacts on health.

1.1 Darwin's view – *An evolutionary story*

Living on our planet requires adaptation to specific environments in order to survive and reproduce. Organisms have evolved to adjust to the conditions of their individual niches and to perfect their ability to cope with its particularities. Most of these habitats have in common that they are subject to a light/dark cycle due to the earth's rotation which limits the presence of light and changes temperature in a daily fashion. A mechanism that could anticipate these predictable daily changes and optimally adjust behavior and physiological as well as biochemical processes to time of day would allow better adaptation (Sharma 2003). Therefore, the internal timekeeping system was established during evolution earliest in prokaryotes and evolved later on also in multicellular organisms, from plants up to complex vertebrates like birds and mammals (Young and Kay 2001, Bell-Pedersen et al. 2005).

The presence of an internal timekeeping system was postulated in eukaryotes in the 18th century. The astronomer deMairan observed that mimosa plant leaves would open and close depending on the presence of sun light. This phenomenon was seen even when the plants were put into constant darkness, pointing to an endogenous property – a self-sustained mechanism that was not induced and driven by light (de Mairan 1729). Almost 200 years later, Bünning showed that this self-sustained timekeeping mechanism was heritable, as period length of bean plants was determined by the one of the mother plant (Bünning 1935). The molecular mechanism that drives this internal biological clock, the so called circadian clock, is nowadays well understood and it is believed that it evolved in parallel to earth's history made: depending on their light/dark schedule, animals have corresponding circadian rhythms in behavior and regulation of body physiology.

Among the five kingdoms of life, circadian clocks are found most numerously in representatives of higher eukaryotes, the kingdoms of Fungi, Plantea and Animalia (Fig 1.1). The molecular clockwork is built on common molecular mechanisms from fungi to

plants or animals. The prokaryotic circadian clock differs in both the proteins and mechanisms implicated. For example, it can function without transcriptional-translational feedback loops (discussed in chapter 1.3.1). Therefore it is believed that the circadian clock among different phyla has multiple origins in evolution (Rosbash 2009).

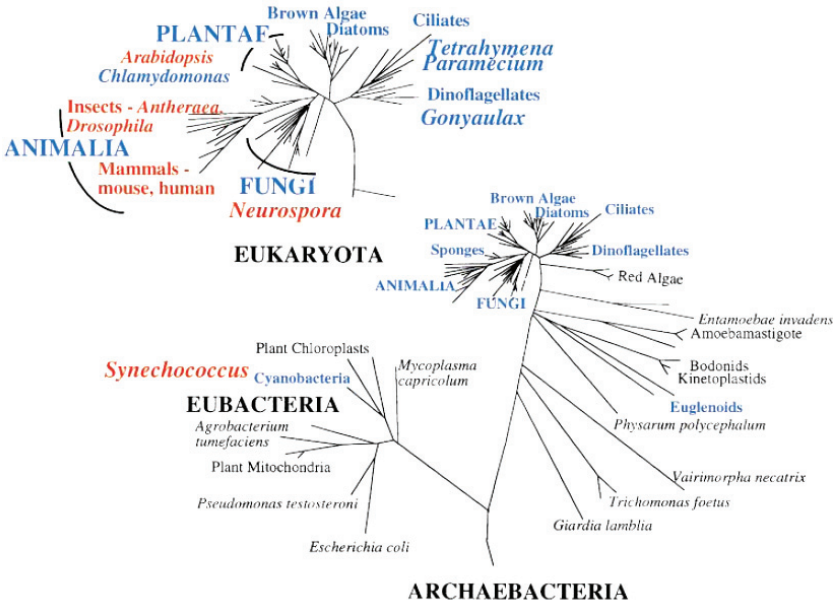

Figure 1.1: The five kingdoms of life and the presence of circadian oscillators (in blue) and studied model organisms (in red). In this phylogenetic tree, lines represent evolutionary relatedness between different phyla and organisms. The different lengths correspond to evolutionary distances measured by mutation rate in ribosomal RNA genes. In the left corner is a magnification of the higher eukaryotes branch. In blue = phyla that possess representatives with a circadian clock. In red = model systems for the study of circadian clock. (From Dunlap 1999)

During the past centuries research in different model organisms across phyla unraveled common principles as well as specific particularities among circadian oscillators of higher eukaryotes based on observation of physiological as well as biochemical parameters.

For several **fungi** it has been shown that the patterning of asexual spore formation by hyphae is produced in a daily rhythmic fashion (Pittendrigh et al. 1959). For example, *Neurospora crassa* has a 22 hour period length (explained in chapter 1.2.2) under constant conditions. Until now *Neurospora crassa* has been the best studied organism of the fungi phyla, though others have followed, like the *Aspergillus* branch (Greene et al. 2003). Light perception in *Neurospora crassa* is an essential trigger for timed mycelia (mass of hyphae) growth that leads to dense areas, visible as bands, of spores (conidia) at the growing front (aerial hyphae). These bands of closely packed spores are laid down in the late night to early morning and alternate with less-dense areas consisting of fewer spores in the other half of the day. Changing the photoperiod length leads to different patterns of bands produced by different numbers of spores and asexual structures, used as a direct measure of the period length in *Neurospora*.

On a daily basis several mechanisms in *Neurospora crassa* are under circadian control: not only visible asexual spore development, but also the key enzymes for stress response and development (Shinohara et al. 2002) as well as proteins involved in detoxification and metal storage (Bell-Pedersen et al. 1996) are thus regulated. Even earlier, it has been shown that daily oscillations in NAD/NADH as well as NADP/NADPH levels indicate a link between the circadian clock and the metabolism of *Neurospora* (Brody and Harris 1973). This subject has been investigated in mammals only recently, as is discussed later (Green et al. 2008, Kovac et al. 2009).

Research in **insects** started with the findings that pupal eclosion (the time point when a fly "hatches") and locomotor activity are clustered to specific times of day in *Drosophila pseudoobscura* a close relative to *Drosophila melanogaster* (Pittendrigh 1967). The understanding of the molecular basis of the circadian core oscillator (Konopka and Benzer 1971) began with the discovery of *period* (*per*), a gene locus that controls for period length. It led also to a model where subpopulations of neurons in the brain are assigned to regulate specific circadian outputs in behavior. For example, the morning and evening locomotor activity introducing the idea of a master clock in the brain (Stoleru et al. 2004). Intriguingly, dissociated parts of the fly body that carried a bioluminescent reporter for the circadian clock continued to oscillate in a circadian fashion even without being connected to the master clock in the brain. This observation established the model of endogenous and self-sustained peripheral clocks in almost all parts of the animal body (Plautz et al. 1997).

The best studied molecular circadian clock in **mammals** is the one of *Mus musculus*. Like in other animals, it has been studied by measuring the overt rhythms of behavior and physiology. The most obvious rhythm is the daily rest/activity rhythm that can be measured in running wheels or with infrared devices to record daily running activity. Other parameters that are measured are hormonal levels in blood or saliva (e.g. melatonin and cortisol). After the finding of the first circadian gene using locomotor mutants in *Drosophila*, the same approach and the use of positional cloning led to identification of the first mammalian circadian clock gene – *circadian locomotor output cycles kaput* (*clock*) a transcription factor (Vitaterna et al. 1994, King et al. 1997). Further genetic studies of the mammalian molecular circadian oscillator revealed other genes (e.g. *period*, *cryptochromes*, *timeless*) and established close parallels between the circadian clocks of *Drosophila melanogaster* and mouse (for review see Bell-Pedersen et al. 2005).

For non-eukaryotic organisms, in the kingdom of protista, the best described circadian oscillator is the one of a **cyanobacterium**, *Synechococcus elongatus*. This "blue-green algae" is a prokaryote that uses the same photosynthetic pathway as eukaryotic cells, algae and higher plants (the Calvin cycle) and is a photoautotroph microbe. Its gene

expression is exclusively regulated in a circadian manner, meaning that all genes are clock-controlled genes (Liu et al. 1995, reviewed in Woelfle and Johnson 2006). Therefore it is not surprising, that in this simple organism without any organelles, the circadian clock is the master pacemaker for all aspects of live, like cell division, nitrogen fixation, amino acid uptake and photosynthesis, as well as respiration (reviewed by Golden et al. 1997). Still there is certainly more to be uncovered in the kingdom of Protista as other bacteria like *Bacillus subtilis* or *Streptomyces* show sporulation patterns that depend upon light conditions (Schauer et al. 1988, reviewed in Golden and Canales 2003).

The evolutionary benefit of a timekeeping system has been more difficult to address scientifically, but some hints exist. Cyanobacteria and plants, for example, perform photosynthesis during the day whereas the decomposition of the toxic side products is carried out during the night. Nitrogen fixation in presence of oxygen that is produced during the day by the photosynthesis machinery would lead to toxic side effects for the cell (Sherman 1998). Compartmentalization in time is one adaptive possibility for simple organisms without organelles (Elías-Arnanz et al. 2011).

Direct evidence for an evolutionary advantage of circadian clocks was demonstrated in cyanobacteria by a study of Woelfle *et al.* in 2004. It was known that mutant strains of the circadian oscillator grew as well as wildtype (Kondo et al. 1994). Moreover, strains that have a clock in resonance with the environment have a better reproductive fitness when kept in competing environment (Ouyang et al. 1998). In Woefle's study, strains of cyanobacteria that had an inner clock adapted to the experimental environment (i.e. that matched the day-night cycle) were co-cultured with either a strain that had no circadian clock or one with a different day length than the environment. The adapted strains overgrew the deficient ones, which even went extinct under this selective pressure. However, this situation changed when the three strains were kept at constant light, where now the genetically modified clock-less cyanobacteria overgrew the ones with a functional circadian clock, indicating that possessing a timekeeping system is associated with a cost and makes only sense in an environment where rhythmic changes occur (Woelfle et al. 2004).

The work of DeCoursey and Krulas in mammals also showed that an intact circadian clock increases fitness. They lesioned the central circadian clock in the brain of chipmunks and released them back into the wild and recorded different aspects of fitness, such as body weight, reproduction and survival. The slight trend toward increased mortality of circadian clock-deficient animals that they found was possibly due to lack of properly timed rest/wake cycle which made the chipmunks more likely to be prey (DeCoursey and Krulas 1998). Other factors such as breeding or body weight were not

varying in these clock-deficient animals, hinting at the extrinsic adaptive advantage of a functional circadian clock.

In other higher organisms such as the fruit fly, period length of different populations has adapted to the latitude (Sawyer et al. 1997), indicating a clear necessity during development and growth to have an entrainable timekeeping system. For plants it has been shown that circadian parameters such as period length, phase, and amplitude vary depending on the environment to maximize fitness (Michael et al. 2003). In *Neurospora crassa* a simple variation in sequence repeats in a circadian core clock gene was shown to be able to modify period length and contribute to phenotypic variations (Michael et al. 2007). These phenotypic variations allow the organism to explore new niches and adapt.

Still, one cannot rule out an intrinsic adaptive value of the circadian clock. The intrinsic advantage of a circadian clock would not depend on a cyclic environment. The work done by Paranjpe *et al.* shows that the fruit fly larvae will continue to hatch in rhythmic manner even if grown for several hundred generations in constant light (Paranjpe et al. 2003). There, possibly the circadian clock has intertwined with the temporal organization of development, so that it is not possible to dispense with it.

In conclusion one can say that an adapted circadian clock enhances fitness in rhythmic environments, at least for some organisms. In addition, depending on the organism, its complexity and physiological as well as developmental organization might furnish an intrinsic adaptive value even in constant environment.

From simple bacteria up to multicellular eukaryotes, most of the phyla developed a timekeeping system and kept it over the past thousands of years of evolution. Thus raises the question when the circadian clock starts to tick in an organism. For a bacterium that does not go through developmental stages the answer is simple. In multicellular organisms like human beings different studies found that physiological parameters known to be regulated by the circadian clock – like heart rate, respiratory rate, movement, and plasma cortisol – already show daily variations in uterus (Serón-Ferré et al. 2001). Using cellular tissue from fetal animals, it has been shown that circadian rhythms were clearly manifested and robustly cycling from embryonic day 18 (E18) (Dolatshad et al. 2010). This suggests that, as in evolution, the circadian clock starts at an early level. This subject is covered in chapter 3.1 that represents the paper *"The circadian clock starts ticking at a developmentally early stage"* (Kowalska et al. 2010).

1.2 Make it tick – *Basic components of a circadian oscillator*

There are three criteria that qualify rhythmic behavior to be based on a circadian oscillator and distinguish it from simple responses to rhythmic external cues: (1) the circadian oscillator continues even in constant environments with an about 24-hour periodicity indicating that it is an intrinsic and self-sustained property rather than driven by an external timing cue. (2) The period length of a circadian clock is robust over a wide range of temperatures within the physiological range and therefore temperature-compensated. (3) The circadian clock can adapt to new environments that have, for example, a changed day length (period length) or different onset of day (phase).

1.2.1 The three building blocks of a circadian system

The three quality criteria for rhythmic behavior described above result from a circadian system that builds upon three parts which are present in each organism with a circadian oscillator and therefore can studied and compared among model organisms.

The first component is an input pathway that tells the actual time of the environment to the circadian timekeeping system, a so called Zeitgeber (Figure 1.2). These timing cues – which might be light, temperature or food availability – bring the circadian system of an organism into synchrony with its respective surroundings by resetting the circadian oscillator to the right phase. These external or exogenous cues are also used by the circadian pacemaker system to readjust and to adapt to seasonal changes, and allow the circadian clock to be flexible and to "entrain" to changed conditions.

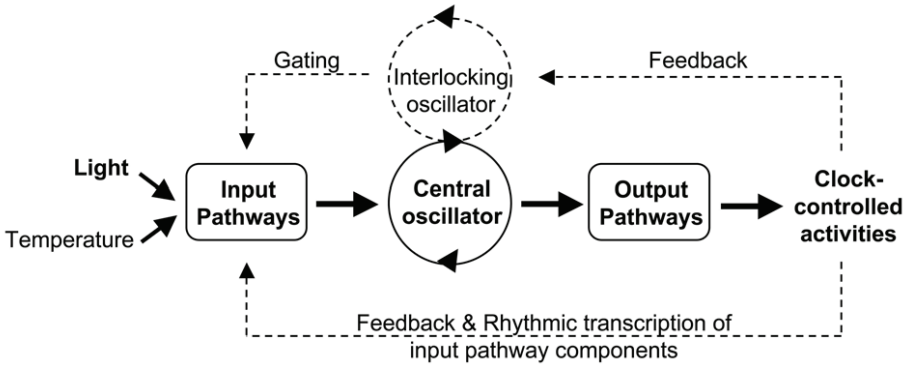

Figure 1.2: The three building blocks of a circadian system. Main components of a circadian clock are shown: Input pathways which determine Zeitgeber time, the central oscillator that determines period length and output pathways controlling physiology and behavior. Dashed lines represent recent findings that overt rhythms like metabolism can feedback and change phase of the central oscillator. (From Kuhlman et al. 2007)

The second component is the circadian oscillator itself, which has a period length of about 24 hours. Period length is the time needed for one cycle (Figure 1.2). As the rhythms are generated endogenously and are self-sustained, the circadian clock also works without environmental timing cues, as shown during constant darkness by the

rhythmic movement of mimosa leaves or in the onset of running wheel activity of a mouse. Under these constant conditions, either Dark/Dark (DD) or Light/Light (LL), the so-called free running period is observed which can be slightly shorter or longer as a 24-hour day.

The third and last element is the output pathways, also called overt outputs (Figure 1.2). They are the visible outputs of the molecular oscillator that manifests itself as rhythmic behavior and physiology (e.g. leaf movement or running wheel activity and metabolism or hormonal levels in the blood stream). Output pathways convey the perceived molecular time information from the circadian clock to the cell and induce the required changes for metabolic or behavioral programs in a body that enable the organism to prepare in advance for the daily changes. These clock-controlled activities can feedback on the central oscillator, as seen in mouse, where the daily exercise in a running wheel reinforces the input pathways, leading to more robust circadian oscillations.

1.2.2 The properties of a circadian oscillator

The parameters describing a circadian oscillator are based on the observation that different behavioral and physiological parameters rhythmically oscillate during the 24 hours of a day. To describe them a sine wave is used as mathematical model. The specific terms used to describe the properties of an oscillator and therefore the ability to compare between different oscillators of individuals and species is described in this chapter. The circadian terms are summarized in Figure 1.3.

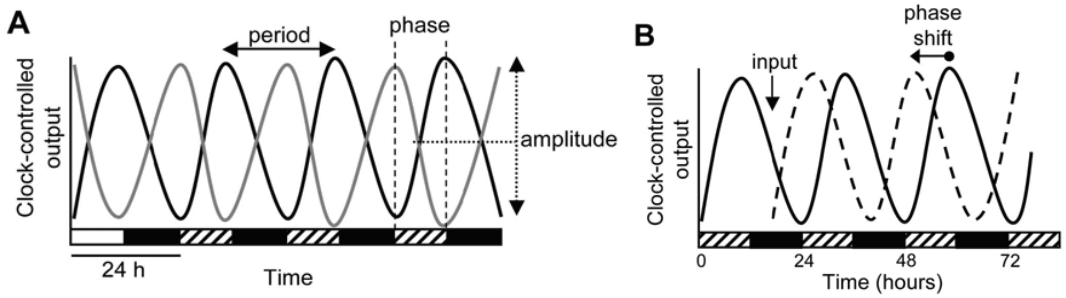

Figure 1.3: Basic properties of an oscillator. (A) Circadian oscillations occur with a 24-hour period length when daily entrained by a Zeitgeber (white/black boxes). Under constant conditions (DD or LL, stripped/black boxes) the oscillator freeruns with a period of about 24 hours. Phase represents a position in the curve relative to a certain time point, with two extremes: peak and nadir. The amplitude depicts the strength of the oscillator and is measured from the midline to peak or nadir. **(B)** By exposing the circadian oscillator to a shifting agent (e.g. light, temperature) the central oscillator will shift its phase to a different position in the curve. (From Kuhlman et al. 2007)

The **period** of a rhythm is measured via the observable circadian behavior of an organism (e.g. running wheel activity, conidiation or leaf movement) or rhythmic transcript levels of a gene. The **period length** reflects the time that the circadian oscillator needs to complete a cycle measured as time between the maxima or minima. The **amplitude** is the

distance from midline to peak or nadir. It reflects the strength of an output of the circadian oscillator and can vary while the period length is unchanged. The period length is measured under “natural conditions”, meaning that the respective clock is synchronized daily by an external timing cue like light or temperature to the local environment (Sharma and Chandrashekaran 2005).

These external timing cues are known as **Zeitgebers** (the german word for time-givers) and entrain the clock on a daily basis to a 24-hour cycle. Therefore the onset of the respective day is defined as Zeitgeber time 0 (**ZT0**). For diurnal as well as nocturnal animals ZT0 it is defined as lights-on (dawn) whereas Zeitgeber time twelve (**ZT12**) is lights-off (dusk).

If this local timing cue changes, for example the onset of light, the circadian oscillator will have to shift its respective starting point of activity (**phase shift**) to the new phase in order to be in synchrony with the external timing cue. The circadian clock is flexible enough to adapt to different day lengths (**entrain**) as well as shift to other points of the cycle (**phases**), but all within certain natural limits and it requires time to do so. This permits the circadian rhythm to entrain if environmental changes occur (e.g. after seasonal migration) and permits a daily resynchronization with the respective environment as the central oscillator does not run with a perfect 24-hour period length.

Under constant conditions, external timing cues are lacking and one can determine the **free running period (FRP)** of an organism. This period length can be slightly shorter or longer than a 24-hour day and reflects the pace of the endogenous circadian oscillator (circa = approximate, diem = day). In the absence of Zeitgeber time, one uses the **circadian time (CT)** for normalizing subjective biological time under constant conditions among organisms with different endogenous period lengths. By convention **CT0** is the subjective dawn and **CT12** the subjective dusk.

The circadian period length is stable over a broad range of physiological temperatures, whereas most biochemical reactions progress quicker in elevated temperatures (e.g. the pyruvate kinase in rats Willmer et al. 2000). The dependence of a reaction on the temperature is measured by the Q_{10} temperature coefficient (**Q_{10} value**). It is the quotient of the reaction rate at the higher temperature to the one 10° Celsius degree below. A **Q_{10} value** of 3 would therefore indicated that the reaction is three times more effective at elevated temperatures. For the circadian FRP this Q_{10} value ranges from 0.85 to 1.4 (Sweeney and Woodland Hastings 1960). The circadian oscillator is therefore **temperature-compensated** (Zimmerman et al. 1968, Gardner and Feldman 1981, Aronson et al. 1994, Barrett and Takahashi 1995, Sawyer et al. 1997, Izumo et al. 2003).

From Cells to Organisms – Different Layers of Complexity in the Organization of the Circadian System

At least in metazoan organisms, for a long time the circadian clock was believed to be an exclusively neuronal phenomenon. In mammals a central clock tissue – the suprachiasmatic nuclei (SCN) of the brain's hypothalamus, the pineal gland in birds and reptiles, and the lateral neurons of Drosophila – was believed to synchronize circadian processes throughout the body via presumably electrical cues to other brain regions. The first evidence that these cues might be primarily hormonal in nature came from pioneering work in the Silver lab, which showed that an implanted SCN encased in porous plastic material could rescue the circadian rhythms of an SCN-lesioned animal (Silver et al. 1996). Other experiments revealed that the basis of this clock is actually cell-autonomous, so to say intracellular rather than intercellular and non-electrical and therefore not dependent on a neuronal network (Welsh et al. 1995).

Soon afterwards, in 1997, the group of Kay showed that in *D. melanogaster* explanted parts of the body possess independent photoreceptive circadian clocks (Plautz et al. 1997). Cell-autonomous circadian clocks were operative throughout the body. Even serum-shocked immortalized rat fibroblasts, isolated over 35 years previously, were observed to have circadian expression of clock genes (Balsalobre et al. 1998). Subsequent experiments with a reporter luciferase construct using the gene promoter of a circadian core clock gene fused to luciferase transgenetically in rats showed that these clocks, in fact, exist in most tissues of the mammalian body (Yamazaki et al. 2000). This finding supported a model where the peripheral tissues are able to generate their own circadian oscillations and are not dependent on the master clock in the brain, but instead are orchestrated by it and receive daily synchronization information (Figure 1.4).

With new molecular biology tools in the second half of lasts century began the search for mutants of the circadian clock and their localization to specific chromosome regions corresponding to specific genes. This combined approach of physiological phenotyping and corresponding molecular localization of the disrupted gene locus by complementation led to the discovery of the first circadian clock gene in fruit flies, the *period (per)* gene (Konopka and Benzer 1971). When *per* was mutated, it resulted in lengthened or shortened timing of pupal eclosion and locomotor activity or the complete absence of it, so called arrhythmia. With this and the following discoveries of other single gene loci in fungi and hamsters it became clear that there was a specific molecular mechanism that drove circadian oscillation. This molecular mechanism was composed of specific genes that when mutated readily disrupted circadian behavior. The molecular mechanism underlying the circadian core oscillator in different model organisms will be discussed in chapter 1.3,

followed by fine tuning mechanisms that trim the circadian oscillator to a 24-hour rhythm in chapter 1.4.

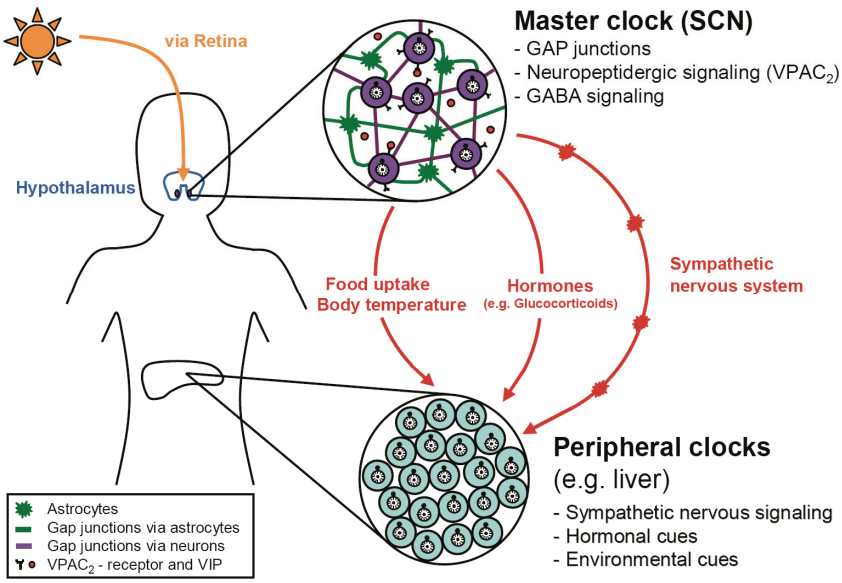

Figure 1.4: Hierarchic Levels of circadian systems organization in mammals. Shown in red are the connections between different body clocks and their influence on other oscillators. In the hypothalamus (blue) the master clock sitting in the SCN is composed of pacemaker neurons (purple) which are interconnected and synchronized by neuropeptidergic signals and/or gap junctions via astrocytes (green) or neurons (purple). Light perceived via the retina and downstream signaling through the retinohypothalamic tract shown in yellow, is responsible for adjusting clock phase in the pacemaker neurons. In peripheral clocks (light blue), as an example the liver clockwork, entrainment is mainly dependent on SCN downstream signaling via the sympathetic nervous system, hormones, and environmental cues (e.g. glucocorticoids). (From Kowalska and Brown 2007)

In a unicellular organism like cyanobacteria or a non-mammal like zebrafish or fruit fly, each cell-autonomous clock is individually light-sensitive and is therefore independently entrained by light, the universal entraining agent (Whitmore et al. 2000). In mammals, however, the synchronization of the circadian system happens in a strictly hierarchical ocular fashion to ensure that clocks throughout the whole organism remain properly synchronized as they lack the ability to sense light themselves (Dibner et al. 2010). First, an external timing cue (principally light) sets the phase in the central pacemaker, the suprachiasmatic nuclei (SCN). This bilateral nucleus contains several thousand independently cycling but locally coupled neurons. Subsequently, the SCN projects its rhythm onto cell-autonomous clocks of similar mechanism in peripheral tissues. The result is synchronous circadian transcription in peripheral tissues with a constant phase delay compared to the SCN (Morse and Sassone-Corsi 2002). The organization and communication of this anatomical master clock with the periphery is in detailed discussed in chapter 1.5.

1.3 The magic within – *Molecular basis of circadian rhythms*

Despite the clear hierarchy in the mammalian circadian system and the slight differences among the clocks in different body parts which will be discussed in chapter 1.5 it is important to focus first on the simple mechanism driving circadian oscillations.

Using bioluminescent reporters it was shown that individual body parts of *Drosophila melanogaster* and skin cells of rats displayed rhythmic output of bioluminescence (Plautz et al. 1997); it became evident that rhythms can be maintained and produced by the smallest subunit of a multicellular organism – the cell itself. The circadian oscillator is based on processes that occur within the simplest organism – i.e. transcription of genes and posttranslational modifications of their output, the proteins. It is important to understand that a single cell is capable of producing circadian oscillations that can be modified by the environment through entrainment cues like light or temperature. This entrainment shifts the output of the circadian clock to the right phase of day. The underlying mechanism of this circadian oscillator is self-sustained and driven endogenously.

1.3.1 As simple as a cell – *The autoregulatory feedback loop, an interplay of transcription and translation*

Each physiological program requires a subset of pathways that have specific proteins which execute the required functions. Besides housekeeping genes that are expressed in all cell types constitutively to permit their growth and survival, other genes require a controlled expression depending on cell type or the developmental stage and in the case of circadian clock genes the time of day. Like in developmental programs the process of timed gene expression during the day is dependent on transcription factors that transform heterochromatin to euchromatin to allow the recruitment of transcription initiation factors and subsequently the transcription machinery itself (Simpson 2002).

In the case of the circadian clock the components are themselves transcription factors and by either being expressed in circadian fashion or having fluctuating protein levels during the day as well as direct competition at the promoter site, they activate or repress their target genes. The precisely timed transcription of clock genes results in fluctuations of transcript levels. Conventionally, these transcription factors are divided into two “limbs”, a positive and a negative one.

First, positive elements in the circadian clock are translated, and assembled as heterodimers. The protein complex shuttles back to the nucleus and activates a second subset of the circadian oscillator, the negative limb. These repressors, once translated, assemble in the cytoplasm to form multimers and shuttle back to the nucleus to repress

the transcription of their own gene loci as well as the transcriptional activators in the positive limb (Dardente and Cermakian 2007).

This molecular model is based upon positive and negative feedback loops of transcription and translation (Figure 1.5 A). Therefore one can observe circadian rhythms on a cellular level by measuring amounts of messenger RNAs of either the positive or negative limb components of the molecular oscillator. Each transcript reaches a peak and a nadir in expression during the day (Figure 1.5 B). Oscillations can also be found in protein levels (Figure 1.5 B). The mRNA and proteins themselves are subject to a cycle of synthesis and degradation in which the mRNA of a respective protein always peaks some hours before the protein (Figure 1.5 B). To maintain these cycles of fluctuations the mRNA as well as the protein need to have a short half-life (Ueli et al. 2003).

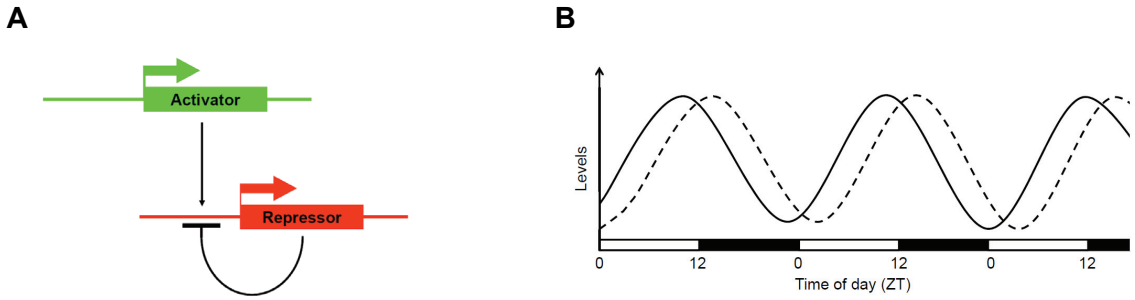

Figure 1.5: The feedback loop model for the circadian clock. (A) A simple gene oscillator involving a transcriptional activator (green) of a repressor (red) that feedbacks its own transcription. **(B)** Circadian expression of mRNA (plain line) or protein levels (dashed line) around consecutive days.

Whether cycling protein levels of all clock components are directly needed to maintain a stable circadian oscillator is still debated. A study by Fan *et al.* for example showed that the levels of one of the repressor protein (CRYPTOCHROME) do not need to vary during a day to be able to rescue the circadian clock mutant phenotype in a *cryptochrome* knockout fibroblast cell model, as their function might be regulated through posttranslational modifications as well as the availability of their heterodimerization partners (PERIOD proteins), instead of their levels their activity could be circadian (Fan et al. 2007).

Similarly, the necessity of the transcriptional feedback loop in the light of the discovery of Nakajima *et al.* is not obligatory for all known circadian oscillators. This group showed that by adding the circadian proteins of the cyanobacterial clock and the necessary cofactors *in vitro* they could build a circadian oscillator that maintained a 24-hour rhythm without the necessity of gene transcription (Nakajima et al. 2005). Findings in a single-celled marine alga (*Acetabularia*) and *Drosophila* where either the cell nucleus was removed or the mRNA of core clock genes was expressed constitutively challenged the transcriptional/translational feedback loop model also in other model organims (reviewed in Lakin-Thomas 2006).

1.3.2 Different kingdoms – different rules

In the next paragraphs different circadian clocks and their regulatory genes and proteins are described in different organisms. Starting with the simplest organism, a bacterium, and working up to mammals we will find the common principle of interlocked feedback loops consisting of transcription and translation based on positive and negative elements. An overview of these regulatory core clock genes and their function is given in Figure 1.6.

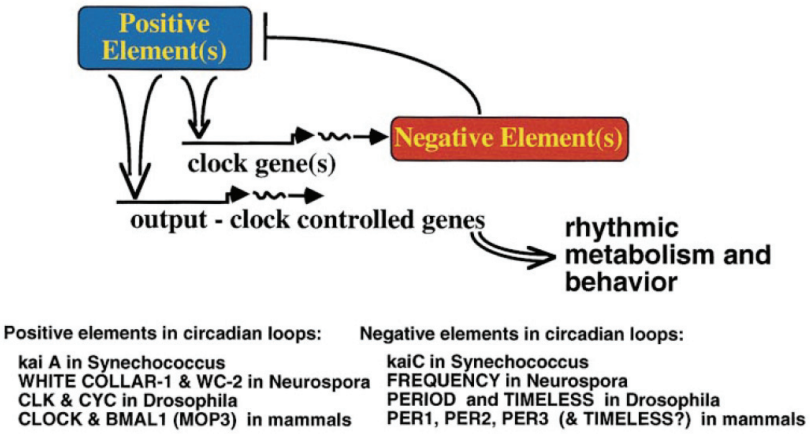

Figure 1.6: Common principles of circadian oscillators among different phyla. The circadian oscillator consists of a simple autoregulatory transcriptional feedback loop of positive and negative regulators of transcription. Beneath the comparison of positive or negative transcription elements in different model organisms. (From Dunlap 1999)

Prokaryotes - *Cyanobacteria (Eubacteria)*

The circadian oscillator machinery The core oscillator consists of the *kai* gene cluster (from the Japanese *kai* for cycle). Their expression is driven by two promoters, the *kaiA* locus and the *kaiBC* locus. The first gives rise to the KaiA protein that is responsible for the positive limb in the oscillator, the latter gives rise to the two proteins KaiB and KaiC (both expressed from a dicistronic mRNA) that represent the negative elements. Knocking out or constantly expressing any of these genes results in arrhythmicity (Ishiura et al. 1998). The *kaiA* gene is rhythmically expressed and peaks around CT9-12, so to say late in the subjective day, and *kaiB* and *C* are expressed sharply at CT12. Whereas KaiA protein levels do not fluctuate during the day, the negative elements KaiB and C have peak levels 4-6 hours after peak mRNA abundance (Xu et al. 2000).

It has been shown by knockout mutant strains that these genes and their protein products engage in a regulatory feedback loop based upon transcription and translation as can be observed in eukaryotes (Ishiura et al. 1998, Figure 1.7), but nowadays it is believed that this feedback is not mandatory to maintain circadian oscillations in cyanobacteria.

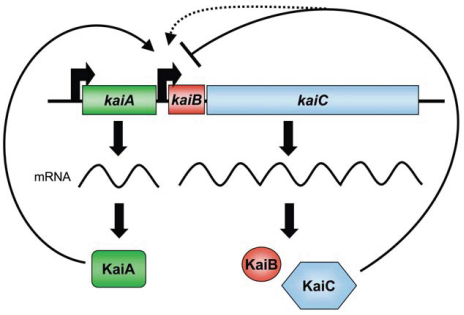

Figure 1.7: The transcriptional/translational feedback loop in *Synechococcus elongates*. The *Kai* gene locus consists of two genetic loci: *kaiA* (green box) and *kaiB/C* (red and blue box). Arrows represent activation of transcription, perpendicular line repressive function on transcription. The dashed line indicates the need for a basal expression level of KaiC to drive expression of the *kaiB/C* gene. (From Mackey 2007)

Particularities of the Synechococcus elongates oscillator The transcription/translation-less circadian clock is based on the observation that KaiC, a member of the RecA/DNAB superfamily of ATPases, can autophosphorylate and dephosphorylate itself in a circadian fashion throughout the day (Rust et al. 2007). KaiC builds hexamers that can be modified in their phosphorylation or dephosphorylation state by KaiA and KaiB (Fig 1.7). Adding the purified clock proteins KaiA, KaiB and KaiC as well as ATP to a test tube enables already self-sustained circadian oscillations for days without the presence of any transcriptional/translational machinery (Nakajima et al. 2005). Therefore the posttranslational modifications of phosphorylation and dephosphorylation shown in figure 1.8 are sufficient to drive the circadian pacemaker in a reliable way (Ito et al. 2007).

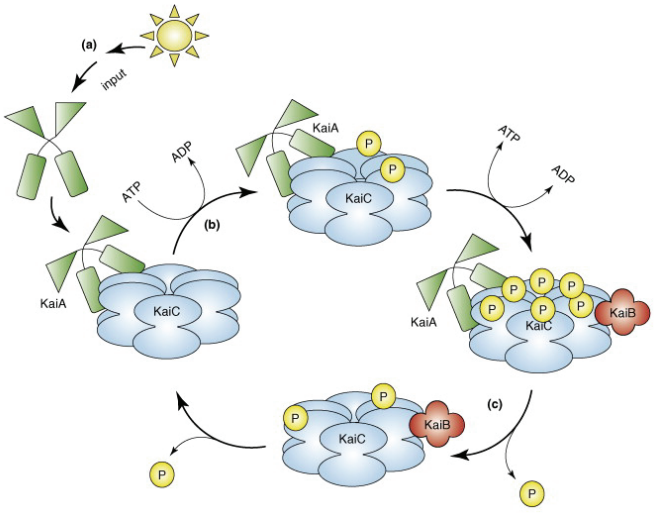

Figure 1.8: The posttranslational circadian oscillator in *Synechococcus elongates*. The transcriptional/translational-less clock is based on KaiC autophosphorylation activity. Hypophosporylated KaiC is bound in the morning by KaiA which stimulates phosphorylation. After reaching a hyperphosphorylated state, KaiC is preferentially bound by KaiB which induces dephosphorylation of KaiC. At this point the cycle starts again. (From Mackey and Golden 2007)

Also, the observation that there are no specific promoter elements in the *kai* genes that are required to retain a circadian regulation of gene expression (Xu et al. 2003, Nakahira et al. 2004) disfavor an oscillator based solely on a transcriptional/translational mechanism. Importantly, this Kai-based posttranslational timing system is temperature-compensated and can be phase-shifted through changes in temperature (Tomita et al. 2005).

Eukaryotes – *Fungi, insects, and mammals*

Transcription is the first step to a functional protein and is tightly regulated by large multi-subunit complexes binding to the promoter. These transcription complexes consist of regulatory elements, DNA binding proteins, and the transcription machinery itself. The regulatory elements are activators or repressors that bind to enhancer sequences and recruit co-regulators which bridge through the TATA binding protein to the promoter site. To this initial complex, basal transcriptional components can bind, and they build up the platform that enables the RNA polymerase to start transcription of the pre-mRNA (Juven-Gershon and Kadonaga 2010).

Whereas DNA binding proteins convey specificity and function as a scaffold during gene expression (Bewley et al. 1998, Halford and Marko 2004), the subsequently recruited co-regulators are the platform at the core promoter for the transcription pre-initiation complex consisting of the respective RNA polymerase and the basal transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH). The co-regulators, either co-activators or co-repressors, are important for the transition from basal transcription levels to adapted transcription levels that suits the cell's need (D'Alessio et al. 2009). They do so by modifying transcription efficiency through recruitment of other proteins that either are capable of remodeling chromatin or modify the basal transcriptional proteins activity.

Neurospora Crassa (Fungi)

The circadian oscillator machinery The core feedback loop consists of three core clock genes: *frequency* (*frq*) which is a transcriptional repressor and *White collar 1* (*Wc-1*) and *White collar 2* (*Wc-2*) (Lee et al. 2000). WC-1 and WC-2 are PAS-domain containing transcription factors that, once dimerized via their PAS domains, form the WHITE COLLAR complex (WCC). The WCC activates the *frq* locus by binding to clock box elements (C-box) in the DNA sequence (Figure 1.9) (Denault et al. 2001).

Several fine-tuning mechanism allow for proper rhythm maintenance through modifying the FRQ protein post-translationally. The FRQ protein homodimerizes and binds to FRQ-interacting helicase (FRH) before shuttling back to the nucleus to repress the WCC mediated transcriptional activation of its own locus through phosphorylation of WCC

(Cheng et al. 2005). This results in a negative feedback that blocks the positive limb of the core clock oscillator (Hong et al. 2008).

In addition FRQ is a target of several kinases (CKI, CKII, PRD-4, CAMK1) (Qun et al. 2003). These protein kinases continuously phosphorylate FRQ, thereby facilitating its interaction with the ubiquitin ligase FWD-1 that targets FRQ for degradation (Qun et al. 2003). The counter players are phosphatases (PP1, PP2A) that dephosphorylate FRQ and allow it to complex with FRH that stabilizes FRQ (Liu 2005).

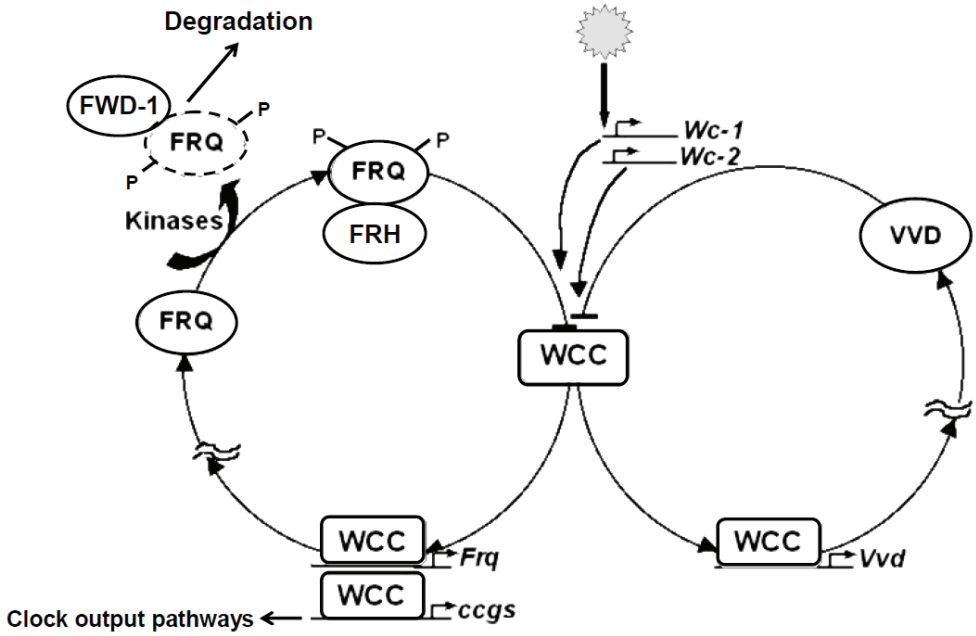

Figure 1.9: The *Neurospora crassa* circadian oscillator. The core feedback loop is displayed in the left circle. Light stimulates expression of *White collar 1* and *2* (*Wc-1*, *Wc-2*) which dimerize via their PAS domain to form the WHITE COLLAR complex (WCC). The WCC activates transcription of *Frequency* and clock controlled genes (ccgs). Phosphorylated FREQUENCY (FRQ) represses the WCC and is also targeted for degradation through binding of the ubiquitin ligase FWD-1 (fainted cycle). On the right side the additional feedback loop responsible for photoadaptation. The *Vivid* (*Vvd*) locus gets activated by the WCC. The VIVID protein shuttles back to the nucleus and represses the WWC-mediated activation of transcription. Blue double circle indicates nuclear envelope. (Modified from Paranjpe and Sharma 2005)

The light input is perceived by WC-1's action within the WCC (Crosthwaite et al. 1997). In addition to being a transcription factor WC-1 is also a photoreceptor for blue light, and the essential factor for light perception and its translation to light responses (Ballario and Macino 1997, Froehlich et al. 2002).

Particularities of the Neurospora crassa circadian oscillator Besides the main feedback loop there is a second one that is regulating photoadaptation through an additional photoreceptor for blue light, VIVID (VVD) that modifies function of WC1 (Schwerdtfeger and Linden 2003). VVD itself is not a core clock protein as a knockout strain still has the same free running period as wildtype. Instead, it influences the proper entrainment to light, and sets the circadian phase correctly (Schneider et al. 2009).

Apparently, there also is another type of circadian oscillator which runs independently of FRQ, the FRQ-less oscillator (FLO), since knockout strains for FRQ can retain residual circadian and non-circadian oscillations (de Paula et al. 2006).

Drosophila melanogaster (Insects)

The circadian oscillator machinery At the core of the oscillator feedback loop there are the positive limb components, CLOCK (CLK) and CYCLE (CYC) that dimerize through their PAS domain, as their *Neurospora* orthologs (Rutila et al. 1998). In addition they possess a basic-helix-loop-helix (bHLH) domain that allows them to bind E-boxes in front of the negative limb elements, *Period* (*Per*) and *Timeless* (*Tim*) (Darlington et al. 1998). PER then binds TIM and together they shuttle back to the nucleus (Gekakis et al. 1995) to shut down their own transcription by competing with CLK/CYC heterodimers at the promoter site (Figure 1.10) (Lee et al. 1999).

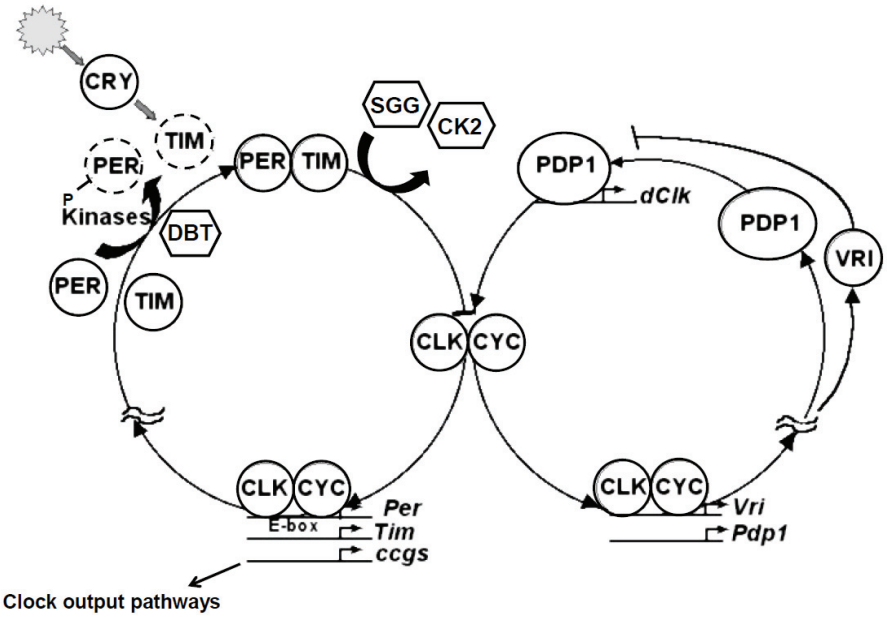

Figure 1.10: The *Drosophila melanogaster* circadian oscillator. The core feedback loop is displayed in the left circle. The transactivators CLOCK (CLK) and CYCLE (CYC) dimerize through their PAS domain and stimulate transcription of *Period* (*Per*) and *Timeless* (*Tim*) as well as clock-controlled genes (ccgs). Once translated and posttranslationally modified by phosphorylation of casein kinase 2 (CK2) and shaggy (SGG), the PER/TIM heterodimer shuttles back to the nucleus and represses CLK/CYC-mediated transcription. PER gets subsequently phosphorylated by DOUBLETIME (DBT) which leads to its degradation (fainted cycle). Light stimulates CRYPTOCHROME (CRY)-mediated degradation of TIM (fainted cycle). On the right side the stabilization feedback loop controlling *Clock* (*Clk*) levels via the activator VRILLE (VRI) and the repressor PAR domain protein 1-epsilon (PDP1ε). Both of them are activated by the CLK/CYC transactivator complex. Blue double circle indicates nuclear envelope. (Modified from Paranjpe and Sharma 2005)

During its time in the cytoplasm, the PER protein gets phosphorylated by DOUBLETIME (DBT), a kinase that is a homolog of casein kinase I (CK1) in mammals (Price et al. 1998). This leads to its degradation and is counteracted by binding of TIM to PER/DBT,

once TIM reaches high enough levels in the cytoplasm (Kloss et al. 2001). This complex is then phosphorylated by two other kinases, casein kinase 2 (CK2) and shaggy (SGG), in order to allow the complex to translocate to the nucleus (Lin et al. 2002). Another way to stabilize PER levels is by dephosphorylation through phosphatase P2A (PP2) (Sriram et al. 2004).

The level of TIM in the cytoplasm is regulated via CRYPTOCHROME (CRY), a blue light photoreceptor similar to WC-1 in *Neurospora*. CRY controls TIM stability via light-dependent degradation (Ceriani et al. 1999). Once TIM is degraded its heterodimer partner PER is no longer able to inhibit transcriptional activation by CLK/CYC in the nucleus, and in addition PER is now subject to degradation via DBT-driven phosphorylation (Price et al. 1998). This enables the activator heterodimer CLK/CYC to drive again the expression of the *period* and *timeless* genes to restart a new cycle.

Particularities of the Drosophila melanogaster oscillator There is a second oscillator intertwined with the core oscillator that regulates levels of CLK by modifying its expression. It involves the activator VRILLE (VRI) and the repressor PAR domain protein 1-epsilon (PDP1ε). Expression from the *Vri* and *Pdp1ε* loci are activated by the CLK/CYC complex (Cyran et al. 2003).

Mus musculus (Mammals)

The circadian oscillator machinery The circadian clock works with feedback loops of activating and repressing transcription factors (Figure 1.11). Activators are the brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1 also known as MOP) and Circadian Locomotor Output Cycles Kaput (CLOCK) whereas PERIODs (PER1-PER3) and CRYPTOCHROMEs (CRY1 and CRY2) are repressors (Shearman et al. 2000). The function of CLOCK in the brain master clock (SCN) can be substituted by its homolog NPAS2 (DeBruyne et al. 2007). The activator complex CLOCK/BMAL1 binds to E-boxes upstream of the repressor genes *Per1-3* and *Cry1/2* (Hardin 2004). In the cytoplasm these proteins can form now the repressor complex PERs/CRYs that upon phosphorylation by casein kinase 1-δ/ε (CK1δ/ε) is able to shuttle back to the nucleus (Lee et al. 2001, Akashi et al. 2002). If the critical level of both binding partners is not reached yet, PERs are readily phosphorylated by CK1δ/ε as well but this time with the consequence that they are targeted for degradation (Lee et al. 2001). Once in the nucleus they bind CLOCK/BMAL1 and suppress their own transcription (Kume et al. 1999).

There exists a second autoregulatory feedback loop that controls the transcriptional activator BMAL1 and is composed of a subset of nuclear orphan receptors (REV-ERBα and β, RORα, β and γ) (Guillaumond et al. 2005). The *Rev-Erbα* gene itself is activated by the BMAL1/CLOCK complex through the E-box motif in its promoter and in the first intron

(Ripperger 2006). They can then modify *Bmal1* expression by binding the specific sequence elements, called ROREs, in the *Bmal1* promoter. Whereas RORα and RORβ are transcriptional activators of *Bmal1*, REV-ERBα is a repressor (Preitner et al. 2002, Akashi and Takumi 2005).

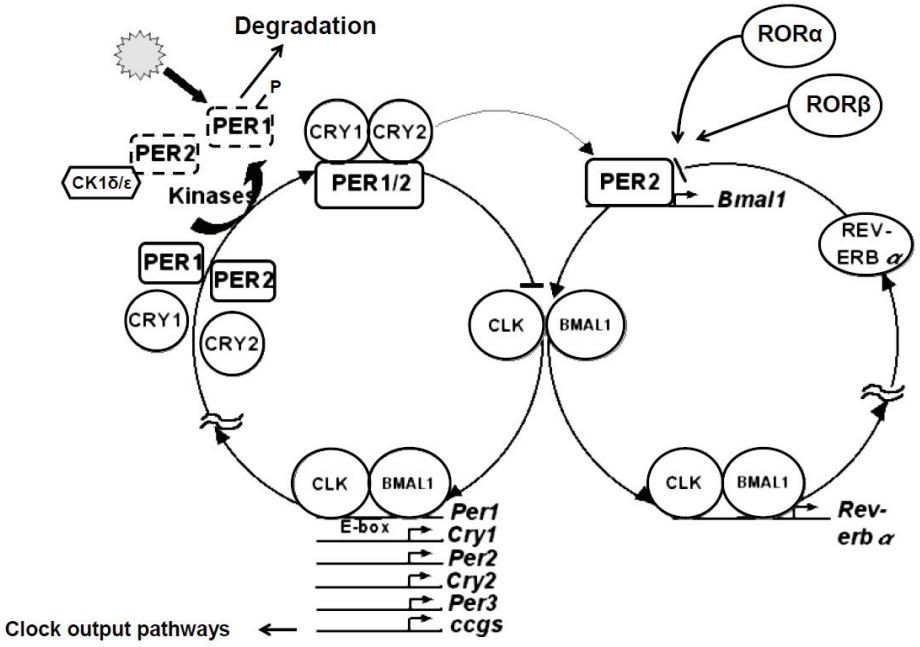

Figure 1.11: The *Mus musculus* circadian oscillator. The core feedback loop is displayed in the left circle. The transactivators brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1) and CLOCK (CLK) dimerize and stimulate transcription of *Period* (*Per1-3*) and *Cryptochromes* (*Cry1-2*) as well as clock-controlled genes (ccgs). Once translated and posttranslationally modified by phosphorylation of casein kinase 1-δ/ε (CK1δ/ε), the PER/CRY heterodimer shuttles back to the nucleus and represses BMAL1/CLK-mediated transcription. PERs get subsequently phosphorylated by casein kinase 1-δ/ε (CK1δ/ε) which leads to its degradation if no dimerization to CRYs occur (fainted cycles). On the right side the stabilization feedback loop controlling *Bmal1* (*Bmal1*) levels via the activators RORα and RORβ and the repressor REV-ERBα. Both of them are activated by the BMAL1/CLK transactivator complex. Blue double circle indicates nuclear envelope. (Modified from Paranjpe and Sharma 2005)

Particularities of the mammalian circadian oscillator The circadian clock repressor complex consists of more than only PER and CRY proteins, it is a multisubunit complex itself containing also coregulators like NONO and WDR5 that influence transcription efficiency through chromatin remodeling (discussed in chapter 1.4.2).

Several subfamilies of nuclear receptor family display circadian expression patterns and link metabolism to the circadian clock control, proposing an explanation of metabolic diseases linked to loss of circadian rhythms (Yang et al. 2006).

Further posttranscriptional and posttranslational regulations of the mammalian circadian clock are discussed in depth in the following chapter 1.4.

1.4 Adaption and precision – *Fine tuning mechanisms*

The circadian core oscillator is based on a delayed negative transcriptional/translational feedback loop in all metazoans as discussed in chapter 1.3. By mathematical modeling (Goodwin 1965, Ruoff et al. 1999), the simple act of feedback: i.e. transcribing a gene whose mRNA shuttles to the cytoplasm, gets translated, and now its gene product enters the nucleus to repress its own transcription would be sufficient to create a self-sustained oscillator. However, when this model was tested *in vitro* by building a synthetic oscillator in *E.coli* the period length was about 2 to 3 hours (Elowitz and Leibler 2000). This first synthetic gene oscillator (called "Repressilator") was built by using known repressor components that were able to transcriptional modify each other's expression once transfected into *E.coli*. The oscillations produced were maintained over a short period (some hours), having the shortcoming that period length among individual cells as well as in one and the same cell showed great variations. In addition the measured amplitude dampened rapidly, pointing to the lack of robustness of this oscillator.

More recently, a new design involving an interlocked positive and negative feedback loop based on transcription (again using *E.coli)* still produced a short period length but was tunable and showed robust cycling. The system also confirmed mathematical modeling: the negative feedback loop was responsible for the oscillations, the positive feedback loop was modifying the period length and contributing to robustness (Stricker et al. 2008). The first synthetic mammalian gene oscillator was built by Chilov *et al.* in 2004 aiming to most closely mimic the *in vivo* conditions. Therefore they used the "original" clock genes of the negative and positive feedback loops and "only" modified the promoter (Chilov and Fussenegger 2004). This system only exhibited only a single cycle of oscillations and therefore did not qualify for modeling the circadian clock. The authors went back and built another mammalian gene oscillator using this time the pristinamycin (PIT) and tetracycline (tTA)-dependent transactivator previously used by Fux *et al.* to control cell growth in mammalian cell lines (Fux et al. 2001). The positive loop consists of tTA being able to activate its own transcription. In addition it activates also PIT gene expression whose product is leading to synthesis of antisense tTA that binds to its sense tTA transcript thereby blocking tTA protein synthesis, thereby accounting for the negative feedback loop. By varying plasmid amounts they were able to show that gene dosage, so to say how much activator or repressor can be made in the beginning, determines the period of oscillations (Tigges et al. 2009).

The conclusions from these *in vitro* studies is that the cell needs built-in mechanisms to dose gene expression or protein abundance to time for delays and be able to trim the circadian clock to a 24-hour clockwork. In mammals, different mechanisms, such as protein abundance, interactions with dimerization partners and subcellular localization,

have been shown to contribute for robust and self-sustained circadian oscillations in cells of the master clock as well as peripheral oscillators. Chapters 1.5.1 - 1.5.3 discuss these mechanisms in detail.

1.4.1 Protein activity, complex formation and protein degradation – *Post-translational modifications*

Once a gene has been transcribed and translated, post-translational modifications can modify its properties to allow the protein to become active (phosphorylation or acetylation), dimerize with other proteins to form a complex or change its subcellular localization. In some cases a series of events, such as formation of a functional complex that now is able to translocate to another subcellular compartment to fulfill its specific task, can allow a protein to perform different tasks depending on its actual binding partner. Some proteins also need to form heterodimers with other proteins or have specific post-translational modifications to protect themselves from degradation that can be induced when the protein gets ubiquitinated or sumoylated and therefore recruits the proteasomal machinery. This regulates a protein's half-life and is especially important in an autoregulated feedback loop.

The transcriptional control in the positive or negative limb of the feedback loop of the circadian clock is executed by heterodimers of transcription factors, BMAL1/CLOCK or PER/CRY respectively. The complexes and the individual partners undergo different states of phosphorylation and dephosphorylation that influences their stability as well as subcellular localization and activity.

The transactivators BMAL1 and CLOCK are phosphorylated in a circadian fashion. This seems to be an important modification, as CLOCK levels do not cycle in circadian fashion (Lee et al. 2001) and constitutive expression of *Bmal1* still produces normal circadian rhythms (McDearmon et al. 2006). Whereas in *Bmal1*- deficient mice CLOCK cannot enter the nucleus (Kondratov et al. 2003), the general disruption of the circadian oscillator for example by mutation of another core clock gene as *Cry*, still displays nuclear CLOCK localization (Tamaru et al. 2003). Phosphorylation of BMAL1 and CLOCK correlate with their ability to dimerize and also with the time point of their highest transcriptional activity (Kondratov et al. 2003). BMAL1 has been shown to be phosphorylated by CK1ε/γ and MAPK *in vitro*. CK1ε/γ positively regulates BMAL1/CLOCK transactivation activity and its deregulation by knockdown or expression of a non-functional protein reduces transactivation activity (Eide et al. 2002). The activity of MAPK seems to repress the BMAL1/CLOCK induced gene expression (Sanada et al. 2002).

Another level of regulation is added to BMAL1 via its SUMOylation, the covalent linking of small ubuitin-related modifier protein (SUMO) to lysine residues. Its mechanism is

similar to the ubiquitin pathway, but it does not predominantly result in proteasomal degradation, but instead can induce changes in localization, protein activity or stability (Hay 2005, Gareau and Lima 2010, Geiss-Friedlander and Melchior 2007). Sumoylation of BMAL1 does not take place when in complex with a mutated CLOCK possessing no transactivational activity, and BMAL1 levels are twice as high when the specific sumolyation-lysine residue is mutated (Cardone et al. 2005). In addition it has been shown that proper sumoylated BMAL1 increases transcriptional performance of BMAL1/CLOCK. This points towards a timed sumoylation of BMAL1 which at first enhances activity of transcription and then leads to ubiquitination and proteasomal degradation (Lee et al. 2008).

Apart from its activity in histone remodeling discussed in the next section, acetylation plays an important role in the activity of BMAL1 and the stability of PER2. The acetylation of BMAL1 by its complex partner CLOCK enables proper recruitment of CRY1 which is present besides PER2 in the competing circadian repressor complex at the promoter site (Hirayama et al. 2007). Recently Asher *et al.* showed that in *Sirt1* knockout embryonic fibroblasts overall PER2 levels are increased as was the acetylation state of PER2, whereas its mRNA levels is decreased. The PER2 protein is deacetylated by SIRT1 (protein discussed in next section) and promotes degradation of PER2 via the proteasomal pathway. Still unknown are the PER2 specific acetylases, but the authors speculate that possible candidates are p300, a coactivator of the BMAL1/CLOCK heterodimer or the HAT activity of CLOCK itself (Asher et al. 2008).

1.4.2 Modulation of transcription – C*hromatin remodeling*

Chromatin structure plays an important role in gene activity. The DNA is packed as “beads-on-a-string”, which are composed of nucleosomes. The nucleosome is built of histones that allow the DNA to wrap around them. The histones can fold into higher orders and build fibers. Loosely packed fibers build the euchromatin which is considered to be accessible to the transcription machinery whereas further compaction leads to formation of heterochromatin that is transcriptionally silent. This equilibrium is controlled by histone modifications that occur at the N-terminal ends of the histone including methylation, acetylation, phosphorylation and ubiquitination (Strahl and Allis 2000, Peterson and Laniel 2004, Kouzarides 2007). Each of these modifications depends on histone type and location of the modified residue within the histone tail. These numerous modifications can result in activation or repression of the surrounding genes. The possible different combinations result in specific remodeling of the genome and led to the “histone code” hypothesis of regulation in epigenetics (Strahl and Allis 2000).

Since it is based on a transcriptional feedback loop, the circadian clock also depends on the open chromatin structure of euchromatin at core clock promoters during the time of day when positive or negative limb components are transcribed. Surprisingly the CLOCK protein possess itself enzymatic activity as a chromatin modifier. Its intrinsic acetylase activity, being a histone acetyl transferase (HAT), can engage in chromatin remodeling via acetylation of histone H3 that is associated with transcription activity (Doi et al. 2006). In addition, it is activating its binding partner BMAL1 by acetylation which is crucial for circadian oscillations (Hirayama et al. 2007).

The CLOCK-mediated acetylation is fine-tuned by SIRTUIN1 (SIRT1), the yeast homolog of SIR2 (silent mating type information regulation 2 homolog). In yeast this histone deacetylase (HDAC) is activated through stressors, such as caloric restrictions via nicotinamidase (PNC1) activity, which occurs when mitochondria switch from fermentation to respiration, which results in reduced levels of nicotinamide and NADH respectively. Activated SIR2 deacetylates specific histones residues at H3, H4, and H1 causing the DNA to coil more tightly. This represses genome instability that otherwise would lead to DNA rings, which permits the cells to continue dividing longer and contributes to longevity (Guarente 2000). Similar effects have been observed for SIRT1 in the mouse (Herranz and Serrano 2010).

In addition SIR2 regulates other transcription factors that are involved in a wide range of critical cellular functions – e.g. Ku70 (a transcription factor that promotes DNA repair and cell survival, Cohen et al. 2004), p53 (a transcription factor that leads to cell death through DNA damage (Luo et al. 2001, Vaziri et al. 2001), and NCoR (a nuclear receptor corepressor complex that affects fat metabolism, inflammation and circadian clock-controlled loci, Alenghat et al. 2008).

The activity of the NAD^+-dependent SIRT1 was found to be regulated in a circadian fashion contemporaneously with acetylation of BMAL1 and histone H3 at circadian promoters. It promotes CLOCK-dependent acetylation of its targets as it resides together with CLOCK/BMAL1 at circadian promoters during the same times of day. Its knockout results in disturbances of circadian oscillations as well as significantly reduced acetylation patterns of BMAL1 and histone H3 (Nakahata et al. 2008). As SIRT1 function is dependent on the cofactor NAD^+, which is produced more prominently during unfavorable metabolic conditions, it is believed to link metabolic activity to genome stability (Bishop and Guarente 2007).

The circadian repressor complex that counteracts the transcriptional activity of CLOCK/BMAL1 has been also implicated in chromatin remodeling. The PER2 protein has been shown to bind to WD repeat-containing 5 (WDR5) (Brown et al. 2005b), a histone methyltransferase adapter important in cell differentiation processes during development

(Gori et al. 2006, Wysocka et al. 2005, Gori et al. 2001). Downregulation of WDR5 resulted in reduced circadian methylation patterns at histone H3 at promoters of clock genes (Brown et al. 2005b).

The importance of histone H3 modifications has been shown for *Per1* and *Per2* promoters where H3 gets acetylated in a circadian fashion coincident with their transcription (Etchegaray et al. 2003). This and other recent findings that the methylation and acetylation of histones follow circadian gene transcription at specific circadian controlled loci promises more chromatin modifiers to be uncovered as finetuning regulators of circadian oscillations (Ripperger and Schibler 2006, Taylor and Hardin 2008, Katada and Sassone-Corsi 2010).

1.4.3 Subcellular localization - *Regulated nuclear import and export*

The circadian core oscillator is based on activation and repression. After translation, both activators and repressors need to shuttle back to the nucleus to activate or repress transcription of their respective target genes. The nuclear entry is timed, as shown for the repressor components PERs and CRYs which arrive in the nucleus with a delay of 6 hours after peak mRNA expression (Hardin et al. 1990, Hastings et al. 1999, Kume et al. 1999, Lee et al. 2001, Yagita et al. 2002). Specific sequences in circadian clock proteins have been described that regulate nuclear import and export, so called nuclear localization signals (NLS) and nuclear export signals (NES) (Vielhaber et al. 2000, Vielhaber et al. 2001, Sakakida et al. 2005, Kwon et al. 2006). The NLS is recognized by an NLS receptor, a complex containing Importin-α and Importin-β that facilitates the import via the nuclear pore (Fried and Kutay 2003).

The NLS of PER1 is used by casein kinase 1 (CK1) to regulate its subcellular localization via phosphorylation. Phosphorylation results in masking of the NLS and the cytoplasmic accumulation of PER1 which leads to delayed nuclear entry (Vielhaber et al. 2000). The PER2 protein has been shown to be dependent on CRY for nuclear localization. In *Cry1/Cry2* mutant fibroblast the protein shuttles between nucleus and cytoplasm. Only in the presence of CRY proteins it starts to accumulate in the nucleus (Yagita et al. 2002). This is promoted in CRY2 via an NLS in its C-terminal, which makes use of the Importin α/β system (Sakakida et al. 2005). Moreover the heterodimer of PER2/CRYs protects both of them from ubiquitination and proteasomal degradation (Yagita et al. 2002). This seems to be dependent on post-translational modifications by GSK3β kinase, as knockdown or inhibition of GSK3β result in inability of PER2 to enter the nucleus (Iitaka et al. 2005). In addition, PER1 is important for nuclear export of CRY1 and CRY2 shown *in vitro* as nuclear injection of PERs and CRYs into *Xenopus* oocytes resulted only for PERs in cytoplasmic localization. Using a dominant negative PER1

protein that cannot shuttle it was also shown that holding CRYs back in the nucleus rendered fibroblasts arrhythmic (Loop et al. 2005).

The components of the positive feedback loop are also regulated during the nucleocytoplasmic shuttling. For BMAL1 it has been shown that its NLS and NES in the N-terminal and PAS domain are essential for accumulation of CLOCK in the nucleus and set the first step for transactivation at the promoters. This would explain why *Clock* expression has no need to be circadian as its product activity in the nucleus is controlled by BMAL1-dependent translocation (Kwon et al. 2006).

The study presented in chapter 3.3 describes a family of RNA-binding proteins as new players in the circadian clock fine tuning mechanisms. The involvement of RNA-binding proteins has been shown before by genome-wide knockdown studies to play a role by disrupting proper circadian oscillations in a bioluminescence assay. The study in chapter 3.2 showed that the running wheel behavior in *Nono*^{gt} knockout mice (lacking the RNA-binding protein NONO) was disrupted only slightly as is observed also in other core clock genes that have multiple family members as *Cryptochrome* genes for example. Single gene deletions cause moderate circadian effects whereas double knockouts result in arrhythmicity (Thresher et al. 1998, Vitaterna et al. 1999). Therefore a bioinformatic search for homologs of NONO was done. The search yield two other proteins that resembled NONO in its domain architecture: all of them share two subsequent RNA recognition motifs (RRM) followed by a NOPS domain (for NONO and PSPC1). The NOPS domain consists of a basic-helix-turn-helix motif followed by a stretch of basic amino acids and is probably binding double stranded DNA and was previously only assigned to NONO and PSPC1 (Staub et al. 2004). The NOPS family members are NONO, the proline/glutamine-rich splicing factor (SFPQ, also known as PSF) and paraspeckle component 1 (PSPC1).

1.5 Keeping up with the master clock - *Peripheral clocks*

The communication between the circadian master clock that resides in the suprachiasmatic nuclei (SCN) and the periphery is essential for the perfect synchronization of behavior and physiology. As peripheral clocks reside in all organs, they have a specific subset of clock-controlled genes (ccgs) under their transcriptional supervision that is required to fulfill a specific body function.

1.5.1 Communication between central and peripheral oscillators

The basic signaling between the core oscillator and peripheral clocks probably involves a mixture of direct hormonal cues such as glucocorticoids and indirect ones such as cyclic

body temperature and food metabolites (Damiola et al. 2000, Le Minh et al. 2001, Stokkan et al. 2001, Brown et al. 2002). Though each of these cues can phase-shift peripheral oscillators without affecting the central clock in the SCN, the elimination of the circadian pattern in any one of these signals does not result in the loss of synchrony in peripheral circadian gene expression. Hence, each of these signals is either redundant or unimportant to circadian synchrony in vivo.

Recent research has even challenged the established hierarchy between the core oscillator and peripheral clocks. For example, the expression of the clock gene *Per1* could be directly induced in the adrenal gland via light in an SCN-dependent mechanism, suggesting the existence of a "shortcut" directly from light to some peripheral clocks. An intact sympathetic nervous system was essential to this process (Ishida et al. 2005). Tissue-specific clock disruptions have confirmed the existence of such direct circuits. Genetic disruption of circadian rhythms in liver results in the abolition of circadian transcription of some liver genes, but not of others – including the clock gene *Per2* (Kornmann et al. 2007). Similarly, the section of the vagus nerve resulted in elimination of oscillations both in *Per2* expression and in acetylcholine receptor protein levels in the respiratory tract (Bando et al. 2007).

Thus, the current working model for circadian clocks is a multi-facet one in which the SCN communicates with peripheral oscillators via several pathways. These peripheral oscillators can in turn directly control circadian genes either via transcription factor cascades or via the same cis-acting elements that control clock genes in general. Finally, some further peripheral circadian gene expression and physiology appears to be controlled not by peripheral clocks, but directly by the SCN via nervous stimuli.

1.5.2 Similarities and differences between central and peripheral oscillators

Considerable speculation has centered on the fundamental nature of clock architecture in SCN neurons and in other tissues. The same basic oscillator components exist in both central and peripheral oscillators, and both are capable of robust cell-autonomous oscillations. Most genetic mutations that affect central oscillator function have similar qualitative effects upon peripheral oscillators (Yagita et al. 2001, Pando et al. 2002). Nevertheless, these effects are often exaggerated in peripheral oscillators, pointing to possible differences. For example, deletion of the *Per1* gene results in a shortening of the circadian period of behavior by one hour, but the period of circadian gene expression in isolated *Per1*$^{-/-}$ fibroblasts is four hours shorter (Brown et al. 2005a).

One possible reason for this difference could arise at the level of the expression of clock components themselves. For example, it has recently been shown that deletion of the important circadian transcriptional activator CLOCK in mice does not abolish circadian

behavioral rhythmicity (DeBruyne et al. 2006). The authors speculate that in the SCN, function of CLOCK can be substituted by the NPAS2 protein (DeBruyne et al. 2007). Since NPAS2 shows a tissue-specific expression pattern, one might suppose that explanted peripheral tissues that do not express NPAS2 would be severely attenuated even though the SCN was not.

Another obvious difference between SCN and peripheral oscillators is that whereas explanted SCN oscillators appear to possess the ability to continue oscillations indefinitely, oscillators in explanted peripheral tissues dampen rapidly (Yamazaki et al. 2000, Yoo et al. 2004). In principal, this experimental observation could arise either through attenuation of clock oscillations in each cell, or via gradually increasing desynchrony among clocks in adjacent cells due to differences in cell-autonomous endogenous period length. Fluorescent or bioluminescent imaging of fibroblast cells in culture firmly supports the latter hypothesis: individual fibroblasts show long-duration circadian oscillations (each of slightly differing period), but fail to synchronize to one another without external stimuli (Nagoshi et al. 2004, Welsh et al. 2004). Although fibroblasts in culture clearly lose synchrony, the same question is less clear in vivo. Confirming the in vitro experiment above, SCN-lesioned hamsters show constant, intermediate levels of clock genes in peripheral organs – an observation that implies cellular desynchrony within each organ (Guo et al. 2006). In contrast, SCN-ablated mice after several days display large phase differences in individual tissues of an animal and among different animals, suggesting the opposite (Yoo et al. 2004).

This discrepancy aside, the clearly superior synchrony among SCN neurons compared to peripheral cells and tissues is likely the result of better intercellular coupling rather than greater clock precision. Dissociated SCN neurons, like fibroblasts, demonstrate significant heterogeneity in period length and phase (Welsh et al. 1995, Welsh et al. 2004). In intact SCN tissue, three clearly defined intercellular coupling methods exist: gap junctions, peptidergic signaling using the VIP neuropeptide and the VPAC2 receptor, and GABA signaling. Elimination of either of these first two pathways results in significant circadian impairments in vivo (Liu and Reppert 2000, Harmar 2003, Long et al. 2005, Maywood et al. 2006).

Current views divide the SCN into at least two functional suboscillators: the dorsal SCN and the ventral SCN. Interrupting the connection between them results in loss of synchrony in the dorsal part of the SCN, but leaves the ventral part perfectly synchronized (Yamaguchi et al. 2003). It is thought that the ventral SCN receives timing information from the retinohypothalamic tract, and subsequently communicates this information to the dorsal SCN neurons. Such a bipartite organization might further stabilize SCN oscillation.

Altogether, experimental evidence and mathematical modeling suggest that intercellular coupling could explain the resistance of the SCN – and by inference circadian behavior – to mutations that more severely attenuate peripheral oscillators of similar molecular makeup (Bernard et al. 2007, Liu et al. 2007).

1.5.3 Peripheral oscillators as probes of circadian clock function

Differences both in clock gene expression and intercellular coupling likely exist between peripheral and central oscillators. Nevertheless, self-autonomous peripheral clocks could provide an important model system for the elucidation of many aspects of clock function that are more difficult or impossible to study in the central SCN oscillator itself, especially in human beings. In principle, peripheral clocks provide two advantages over the study of the whole organism or of the central clock in the SCN: accessibility to experimental manipulation, and availability in homogenous large quantities. Multiple laboratories have exploited these aspects for both biochemical and genetic studies into the mammalian circadian oscillator.

By labeling the clock protein PER1 with peptide epitopes and then expressing it in fibroblasts, our laboratory was able to purify a PER1-containing protein complex that contained CRYPTOCHROMES, as well as two other novel proteins, WDR5 and NONO. Fibroblasts were then used as easy model systems in which to study the function of these two proteins. RNA interference-based knockdown of NONO protein levels demonstrated NONO to be essential to circadian rhythms in these cells, and knockdown of WDR5 demonstrated that it was necessary for histone methylation at circadian clock loci (Brown et al. 2005b).

Of course, the observation that WDR5 and NONO are important to clock function in fibroblasts does not permit an immediate generalization for the whole organism. The final “acid test” of validity remains the analysis of the whole organism. Usually, this test is carried out via a mouse knockout model. Such a knockout is generated by homologous recombination in embryonic stem (ES) cells, which are then injected into a mouse blastocyst to create a chimera – a time-consuming and costly process. Since these ES cells are pluripotent, their differentiation into other cell types that exhibit circadian rhythmicity permits the rapid screening of generated cells for circadian phenotypes, at least if the targeted gene has a phenotype at the heterozygous or hemizygous level. Coupled with “gene trap” approaches to generate nonfunctional alleles, such an ES cell differentiation approach could be used as a rapid screen for new X-linked clock genes (Kowalska and Brown, unpublished observations).

Fibroblast oscillators have also been used as functional tools to identify the underlying mechanism of human mutations that cause circadian disorders. For example, Familial

Advanced Sleep Phase Syndrome has been mapped in one family to a point mutation in the *Per2* locus (Toh et al. 2001). By expressing the mutant allele in fibroblasts, Vanselow and colleagues were recently able to characterize the nature of this defect at a molecular level, as well as to recapitulate the advanced phase of the behavioral phenotype of this mutation by measuring the transcriptional phase of FASPS fibroblast cells under entrained conditions (Vanselow et al. 2006).

1.5.4 Outlook: Potential uses of peripheral clocks to characterize human disorders

Using peripheral cells to verify or study human phenotypes could potentially impact patient care and diagnosis in a clinical setting. Although the human circadian oscillator has been characterized extensively at a behavioral level, the difficulty and cost of maintaining subjects under controlled conditions to effect these measurements prevents their widespread use. Easily-available peripheral tissues (blood, skin, hair) could provide a useful proxy. Primary cells from these tissues can be infected with lentiviral or adenoviral reporter vectors that permit bioluminescent readout of circadian gene expression, thereby enabling the investigator to monitor different properties of the molecular clock and characterize its function (Brown et al. 2005a).

For such studies to be possible, it is important to establish the relationship between circadian properties measured in peripheral tissues such as fibroblasts and those measured via behavior. Although initial studies have shown excellent correlations between behavior in mice and the molecular properties of fibroblasts (Fig. 1.12), further studies in human beings are necessary to validate these conclusions. Fibroblast period length per se is influenced by culture conditions such as temperature and the concentration of serum in their growth medium. Nevertheless, cells displaying short and long period lengths seem to retain their relative values under all conditions. Thus, although comparisons of values from different laboratories may prove problematic, the assay as a whole shows great promise.

Specifically, peripheral oscillators as a model system might permit screening of patients with sleep disorders to determine which are due to molecular defects in the circadian clock. When peripheral cell cultures are kept under constant growth conditions, an estimate of free-running period length can be obtained. By placing them in entrained conditions – e.g. 24-hour temperature cycles – one can then look at entrained phase. It will be interesting to see how both these properties correspond to behavior in human subjects. Finally, by using these properties as quantitative traits in human pedigrees or populations, genetic linkage or association studies could be possible, enabling the discovery of modifier loci for human chronotypes.

Recent studies all highlight the extent to which circadian clocks impact not only behavior, but also cellular processes such as cell division and metabolism. Peripheral oscillators could also provide an excellent model system in which to study these phenomena. For example, the involvement of the circadian clock in DNA damage check point control, whose dysregulation leads to cancer (Collis and Boulton 2007). Doubtless, further investigations will ascertain not only the potential but also the limits of this exciting model system.

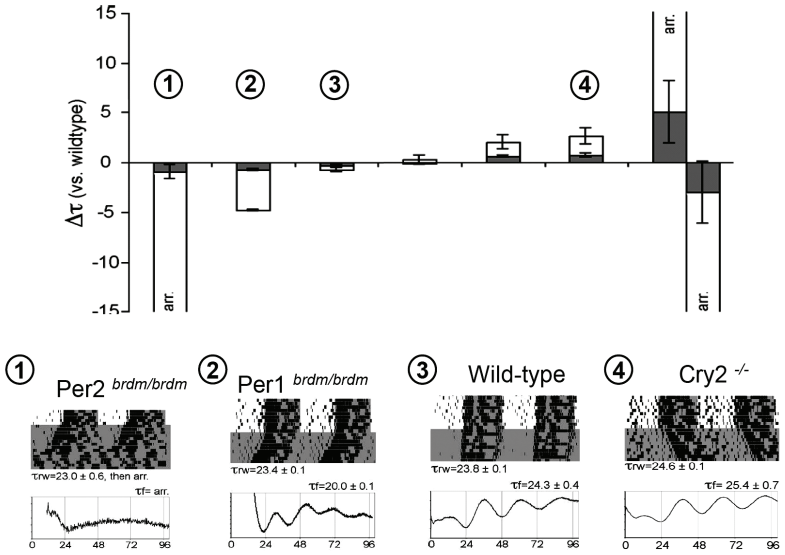

Figure 1.12: Comparison of period length between central and peripheral oscillators. Genotypic variation of period lengths in different mutant backgrounds. The variation of circadian period length measured from fibroblasts (white bars) is compared to behavioral period length measured via running wheel activity data (light grey bars), expressed as difference in hours from the 24-h light cycle. Genotypes shown in top panel from left to right: *Per2*$^{brdm/brdm}$, *Per1*$^{brdm/brdm}$, *wild-type*, *Cry2*$^{+/-}$, *Cry2*$^{-/-}$;*Per1*$^{brdm/brdm}$, *Cry2*$^{-/-}$, *Per2*$^{brdm/brdm}$;*Cry2*$^{-/-}$. In the lower panel representative running wheel actograms from individual animals are shown. (Figure adapted from Brown et al. 2005a).

Daily Business – the Circadian Clock and Body Functions

In most organisms, circadian rhythms play a key role in the regulation of numerous aspects of physiology and behavior. The behavioral rhythms manifest themselves in sleeping, food seeking, predator avoidance, cognitive performance, as well as mating and reproduction (Moore-Ede 1982). These behavioral rhythms have their origins in physiological or cellular programs.

The physiological rhythms controlled by the circadian clock include rest-activity cycles, hormone level variations in the blood, body temperature, renal activity, cardiac function (heartbeat, blood pressure) and all aspects of food processing and metabolism, from gastric emptying to detoxification (Ripperger and Schibler 2001, Merrow et al. 2005, Takahashi et al. 2008).

The malfunction of the circadian clock was found accompanying diverse disease pathologies in human beings. Our modern society imposes with its social time cues (e.g. working and leisure schedule) deviations from the natural given light/dark cycles (Wittmann et al. 2006). This could result in misalignment of the inner circadian clock with the respective environment and leads to continuous mis-entrainment. The outcome is loss of productivity and decreased cognitive performance, as well as enhanced risk of errors and accidents (Gold et al. 1992, Lockley 2007, Santhi et al. 2007, Kyriacou and Hastings 2010, Wright et al. 2011). But not only mental deduction consequences follow from disrupted circadian clocks. There are also health risks such as gastrointestinal, metabolic and cardiovascular disorders, sleep disorders, as well as increased risk of depression and cancer (Scheer et al. 2009, Akerstedt and Wright 2009, Wirz-Justice 2006, Emens et al. 2009).

When comparing day workers to night workers or those on a rotating-shift one can observe an increase in the risk to develop breast or prostate cancer as a direct function of how long the person was exposed to the aberrant social timing cues (Davis et al. 2001, Schernhammer et al. 2003, Kubo et al. 2006, Davis and Mirick 2006).

Obesity is increasing over the past decades and has grown to an epidemic. With it comes type 2 diabetes and cardiovascular diseases that are among the top 10 causes of death in our society (WHO statistics[1]). This rise of the metabolic syndrome, a combination of health impairments that increase the risk of developing cardiovascular disease and diabetes (NIH[2]), seems in part to derive from a chronic misalignment between rest/activity and fasting/feeding cycles (Allison et al. 2007). Its pathogenesis has been linked to altered cirdadian rhythmicity as well as sleep patterns. These perturbations lead to obesity, diabetes mellitus, cardiovascular disease, thrombosis and inflammation (Maury et al. 2010).

The impact of mood disorders became in the last century an important factor in overall well-being of a person. The circadian clock controls many hormones and metabolites within the endocrine system (Hastings et al. 2007) and therefore it was predictable that mental diseases would have a disturbed circadian profile of hormone output. Different studies showed that people with mood disorders, such as depressions, had lost circadian rhythms not only in body temperature and rest-activity cycles (insomnia) but also levels of hormones – i.e. melatonin, thyroid stimulating hormone (TSH), noradrenalin and serotonin were no longer secreted in a circadian fashion (McClung 2007, Mendlewicz 2009, Schulz and Steimer 2009). Whether these mental disorders are a direct cause of disturbed sleep

[1] http://www.who.int/mediacentre/factsheets/fs310/en/index.html
[2] http://www.nlm.nih.gov/medlineplus/metabolicsyndrome.html

patterns, or are enforced due to aberrant social timing cues, or have their source in another genetic locus is still to be seen (Cermakian and Boivin 2003).

Disrupting the homeostatic network controlled by the circadian clock by either actively counteracting the clock-determined restrictions (like sleep-wake cycles) or genetic mutations in core clock genes – will lead to abolitions of the homeostatic control between the different physiological programs and biochemical pathways, which could facilitate diseases as cancer, metabolic disorders and infertility (Fu 2002, Miller et al. 2004, Takahashi et al. 2008). In the above discussed diseases related to aberrant circadian clock physiology it is still unclear, however, whether these phenotypes are related directly to the clock or to other clock-independent functions of circadian clock genes.

In organs like liver, heart, SCN and pineal gland up to 10% of the whole genome is transcribed in a circadian fashion, as was shown by gene expression profiling using microarrays (reviewed in Delaunay and Laudet 2002). In comparison to organs fibroblasts have a significantly lower number of cycling genes, up to 2%, probably due to lack of SCN signaling and therefore less robust cycling and increased dampening after serum shock (Grundschober et al. 2001). The clock-controlled genes (ccgs) are under direct control of the circadian clock via transcription. Interestingly the subset of clock-controlled genes varies from tissue to tissue and two different tissues like heart and liver have very little commonly clock-controlled genes (Panda et al. 2002, Storch et al. 2002). Those in common participate in related pathways and show general function – e.g. amino acid metabolism in liver or G-protein coupled signaling in heart (Storch et al. 2002).

In the past years specific molecular links were found that couple metabolism and circadian clocks (Green et al. 2008). For example, Per2 is found to rhythmically bind the promoters of nuclear target genes *in vivo* and interacts with nuclear receptors like PPARα and REV-ERBα (Schmutz et al. 2010). This subgroup of nuclear receptors has been shown to directly regulate key players of the rhythmic control of the energy, glucose and lipid metabolism (Yang et al. 2006). Therefore evidence is accumulating that metabolism could be under the control of the circadian clock, and the two may feedback on each other.

The impact of cell cycle control by the circadian clock will be described in chapter 1.6. In our western society, cancer became besides cardiovascular diseases one of the main causes for death (WHO[3]). In chapter 1.7 it will be outlined which role the circadian clock might play in cancer pathology and how this links to the new findings in Chapter 3.2 that were submitted as the paper “NONO couples the circadian clock to the cell cycle” (currently in review), which shows that hyperproliferation as well as loss of cell cycle gating lead to loss of intact wound healing in NONO-depleted mice.

[3] http://www.who.int/mediacentre/factsheets/fs310/en/index.html

1.6 The cell cycle and its control

Control principles similar to those of the circadian clock can be found in the cell cycle program. It runs also on timed transcription steps that lead to production of proteins which are subject of posttranslational modifications and whose activity ends by timed degradation (Oikonomou and Cross 2010, Johnson 2010).

Built in are autoregulatory feedback loops that ensure each step to be tightly regulated (Takuwa and Takuwa 1996, Hunt and Sassone-Corsi 2007). Still, the cell cycle is not compensated for temperature changes (Lopez-Saez et al. 1966), and changes in nutritional state also lead to variation in cell cycle length (Fantes and Nurse 1977, Hartwell and Unger 1977).

The cell cycle duration can vary from several minutes in fly or frog embryos and bacteria (Shermoen and O'Farrell 1991, Siegal-Gaskins and Crosson 2008) to more than an hour in budding yeast (Di Talia et al. 2007) and up to 55 hours in developing retinal cells of rats (Alexiades and Cepko 1996). On average a mammalian cell will spent 24 hours to complete a cycle (Cooper 2000). During the past decades, evidence accumulated that the cell cycle is timed by the circadian clock and specific cell cycle stages are gated to specific times of the day (Canaple et al. 2003, Rensing and Goedeke 1976). Studying the cell cycle in a unicellular marine dinoflagellate (*Gonyaulax polyedra*) one can observe that 85% of all cell divisions are carried out during the transition from night to day (Sweeney and Hastings 1958). Also in mice the peak time for DNA synthesis that follows a 24-hour rhythm in different tissues is occurring at dawn (Scheving 1981, Scheving et al. 1978).

In each cell division a mother cell divides and gives rise to two genetically identical daughter cells. This process is time and energy intensive and therefore is tightly linked to the metabolic state of the surroundings of the cell itself. If nutrients are deprived or the favorable growth hormones absent, the cell will remain in its default state, the G0 phase. In this quiescent state the cells maintains its own survival and tissue-specific functions (Norbury and Nurse 1992). If the necessity arises to replicate, as for example in the case of wounding or when outworn cells have to be replaced (e.g. in the gastrointestinal tract), the cell will receive specific signaling from the environment to enter the first of the four cell cycles stages, the gap 1 (G1) phase (Norbury and Nurse 1992). In this phase the production of proteins for DNA replication machinery and cell growth are induced. The next step is to replicate the DNA that has to be equally distributed between the daughter cells during the DNA synthesis (S) phase (Enoch and Nurse 1991, Norbury and Nurse 1992). Once this is accomplished, the cell has to express and synthesis during gap 2 (G2) phase the proteins that will execute the cell division and prepare metabolites (Norbury and Nurse 1992). Finally, in the mitosis (M) phase the sister chromosomes are separated with

the spindle apparatus to the respective newly formed cell (Lewin 1990, Enoch and Nurse 1991, Ohi and Gould 1999, Nurse 2000).

To proceed through the cell cycle the activity of cyclins which regulate the activity of their specific cyclins dependent-kinases (CDKs) is required (Hunt 1991, Pines 1994). While expression of each cyclin is timed to a specific time slot during cell cycle transition, CDKs are expressed continuously, but need to bind their corresponding cyclin to become active and therefore are regulated in a temporal manner (Maller 1991, Coudreuse and Nurse 2010). Each single transition needs precise feedback about overall progress – for example of the DNA replication in the S Phase or DNA damage during replication – to time the next step. The cell uses autoregulatory feedback loops, so-called cell cycle check points (Figure 1.13), which sense abnormalities during cell cycle progression, such as breakage of DNA strands or incorrect alignment of chromosomes during mitosis (Enoch and Nurse 1991, Lukas et al. 2004). The cell cycle check points are named after the transitions they control: the G1/S, S phase, and G2/M checkpoint (Hartwell and Weinert 1989, Rieder and Khodjakov 1997, Walworth 2000, Nojima 2004).

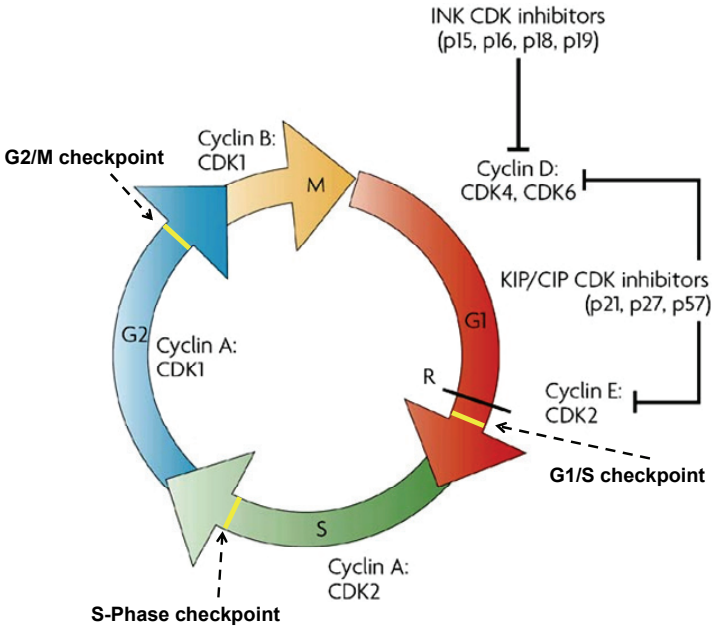

Figure 1.13: The cell cycle and its checkpoints. Central circle indicates different phases of the cell cycle: initial growth (G1 – red arrow), DNA synthesis (S – green arrow), the gap phase (G2) and mitosis (M - yellow arrow). Each phase has a specific regulatory cyclin expressed (Cyclin D, Cyclin E, Cyclin A, Cyclin B) which binds a specific cyclin-dependent kinase (CDK) – CDK4, CDK6, CDK2, CDK1. CDK activity is regulated through different CDK inhibitors modifying progression through the cell cycle (INK CDK inhibitors and KIP/CIP CDK inhibitors). Yellow bars within arrows mark cell cycle checkpoints at transitions. The restriction point (R – black bar) is the critical point of cell cycle progression: passing this point the cell cycle is irreversible. (Modified from Dehay and Kennedy 2007)

The cell cycle checkpoints use different DNA damage response (DDR) pathways that engage in repair, blocking cell cycle progression and signaling progress throughout the

cell cycle to guarantee genomic stability (Nojima 2004, Bartek et al. 2004, Chow and Poon 2010). To assure the quality and integrity of the new genetic material they are also able to trigger apoptosis, programmed cell death if damage is irreparable (King and Cidlowski 1995, Niida and Nakanishi 2006, Clarke and Allan 2009). If any of these cell cycle regulators are deregulated or mutated this results in uncontrolled cell growth and can lead to mistakes in DNA distribution or duplication that are now passed on to daughter cells (Motoyama and Naka 2004, Shimada and Nakanishi 2006, Ishikawa et al. 2006). This might lead to formation of cell line precursors that hyperproliferate and can induce mal-development or tumorigenesis (Nojima 2004, Poehlmann and Roessner 2010).

The cell cycle was shown previously to be timed by the circadian clock. Several key players of the cell cycle checkpoints are circadian clock-controlled genes (ccgs), genes that are under the direct transcriptional control of one of the core clock proteins (Borgs et al. 2009). In addition, it was shown that mice with a disrupted clock became more susceptible to develop cancer (Sahar and Sassone-Corsi 2009, Borgs et al. 2009, Johnson 2010) and this link will be discussed in the next chapter.

1.7 Circadian rhythms and cancer

Circadian rhythms are profoundly regulating physiology in animals and from microarray gene expression profiling it appears that they regulate rate-limiting steps in tissue-specific physiological programs (Panda et al. 2002). As discussed before the risk of developing cancer is higher in shift workers as well as in mice with a dysfunctional circadian clock (Davis and Mirick 2006, Fu and Lee 2003). In this section the molecular and physiological links between circadian clock and cell cycle that may promote this elevated tumorigenesis will be highlighted.

For a tumor three things are essential to become a successful invader (Hanahan and Weinberg 2000): (1) It must overcome the natural limitations of cell division due to cell cycle restrictions to proliferate indefinitely and at any time, so-called immortalization. (2) Upregulated energy supply has to be guaranteed to get energy fast and in constant supply. Therefore a cancer preferentially uses anaerobic glycolysis for ATP production instead of the mitochondrial oxidative phosphorylation pathways of the Krebs cycle coupled to the electron transport chain (Warburg 1956). (3) It has to overcome tissue barriers and travel within the body to “colonize” other tissues. The ability for invasion makes a cancer almost impossible to cure as metastases form in most parts of the body and serve as niches for new cancer cells. These three points mark a disruption of natural homeostasis and allow cell to transform into a cancer cell line.

To overcome the natural limitations in cell division the cancer will have to override cell cycle check points discussed in the previous chapter. In *Per2* mutant mice several key cell

cycle regulators (*Cyclin D1*, *CyclinA* and *c-Myc*) previously shown to be under circadian transcriptional control were misregulated (Fu et al. 2002). The **G1/S checkpoint** control protein Cyclin D1 lost circadian expression and was derepressed at time points in the day were it is normally silent in wildtype mice. *c-Myc*, an important oncogene, shows a dramatic increase over the whole day when PER2 is gone. *C-Myc* was shown to be repressed by the transcriptional activators BMAL1/NPAS2 and the elevated levels in *Per2* mutant mice suggest that PER2 is essential for the BMAL1/NPAS2-mediated repression (Fu et al. 2002) (Figure 1.14). The overexpression of C-Myc was previously shown to lead to abnormal proliferation resulting in DNA damage, accumulation of mutations and therefore genomic instability (Prochownik 2008).

Another BMAL1/CLOCK(NPAS2) transcriptionally regulated cell cycle gene is *Wee1* (Figure 1.14). Both *c-Myc* and *Wee1* have been shown to possess E-boxes that are under BMAL1/CLOCK(NPAS2) transcriptional control (Matsuo et al. 2003). *Wee1* is overexpressed when PER1 levels are high and during that time of day entry into M phase is suppressed (Matsuo et al. 2003). WEE1 is a **G2/M transition checkpoint** kinase that is competing with CDC25C for the phosphorylation state of CDC2/CyclinB1 which determines progression from G2 to M phase (Perry and Kornbluth 2007, Cardone and Sassone-Corsi 2003). The G2/M transition checkpoint is also under control of another circadian protein, human TIM, the ortholog of *timeless* in *Drosophila*. TIM was found to bind the DNA damage control (ATR-ATRIP) complex which acts as a repressor of CDC2/CyclinB (Unsal-Kaçmaz et al. 2005) (Figure 1.14).

The **ATM/ATR pathway** is an important initiator of DNA damage response (DDR) pathways. Both key players are activated by DNA breakage, the ataxia telangiectasia mutated (ATM) kinase by double-strand breaks and the ataxia telangiectasia and Rad-3-related kinase (ATR) by single-strand breaks (Hurley and Bunz 2007, Bartek and Lukas 2007, Smith et al. 2010).

ATM phosphorylates Serine/threonine-protein kinase 2 (CHK2) which sets off the CHK2 activated pathways (Figure 1.14) among which is the p53 pathway. CHK2 is an inhibitor of Cyclin D1 and its activity results in arrest of cell cycle progression in G1 phase (Reinhardt and Yaffe 2009, Brooks and Gu 2010). The ATM/CHK2 complex has been found to be bound by PER1. The overexpression of PER1 leads to decrease of cell proliferation in human cancer probably through increased activity of the ATM/CHK2 complex (Gery et al. 2006).

ATR on the other hand acts on Serine/threonine-protein kinase 1 (CHK1) and by phosphorylating CHK1 and actively binding ATRIP it forms the ATR-ATRIP complex. This complex was found to repress CDC2/CyclinB1 and arrest cell cycle progression in G2 phase (Syljuåsen et al. 2005).

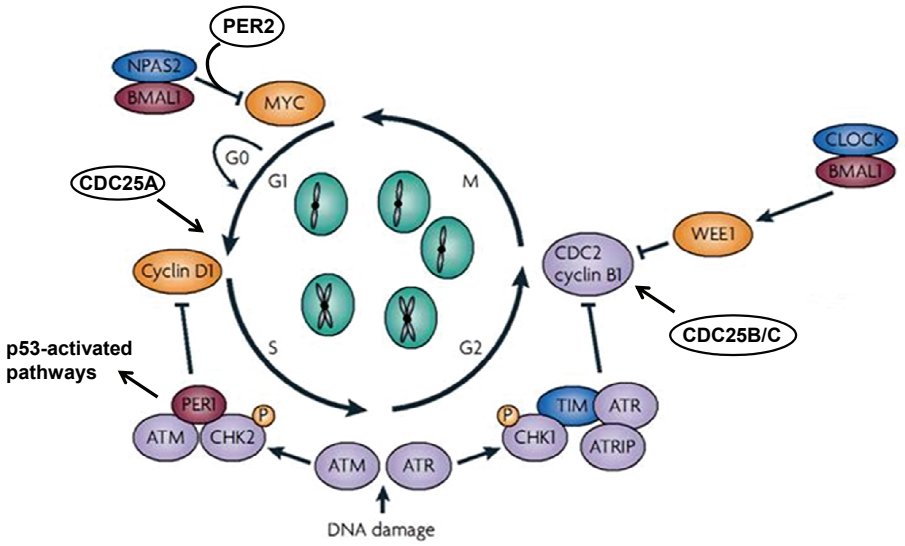

Figure 1.14: Circadian clock control of the cell cycle. Cycle represents the four stages of the cell cycle (G1, S, G2, and M). The ATM/ATR pathway regulates Cyclin D1 at the G1/S checkpoint and Cyclin B1 at the G2/M checkpoint involving two circadian proteins (PER and TIM). *c-Myc* and *Wee1* are controlled at the transcriptional level through BMAL1/CLOCK(NPAS2). Multiple CDC25 phosphatase family members are participating in cell cycle check points. Whereas *cdc25B* and *cdc25C* are involved in the G2/M transition and M phase checkpoints, the *cdc25A* phosphatase regulates cell cycle transition from G1 to S phase (Boutros et al. 2007). (Modified from Sahar and Sassone-Corsi 2009)

The **p53 protein** regulates multiple cell cycle checkpoints and is a classical tumor suppressor, a gene that protects the cell from tumorigenesis. Once activated it leads to cell cycle arrest, DNA repair onset and when irreparable damage occurs, to apoptosis (Levine and Oren 2009). ATM patients display hypersensitivity to DNA damaging factors as ionizing radiation an UV light and are predisposed to tumorigenesis (Derheimer and Kastan 2010) a phenotype that is also observed in *Per2* mutant mice (Fu et al. 2002). Lack of PER2 prevents p53-driven apoptosis of cells that accumulated irradiation damage in their DNA pointing to tumor suppressor function (Fu et al. 2002). The finding that PER1 overexpression in cancer cell lines that are exposed to ionizing radiation increases apoptosis (Hua et al. 2006), whereas the knockdown of PER1 protects the cells and its interaction with ATM/CHK2 qualifies it as well as a tumor suppressor gene (Gery et al. 2006).

In summary there exist multiple circadian control points upon DNA damage response pathways (DDR) and regulation of two cell cycle checkpoints, Cyclin D1 and Cyclin B1. Circadian control is executed upon them either directly through binding to a circadian core clock protein or circadian transcriptional regulation (Figure 1.14) (Sahar and Sassone-Corsi 2009).

The accumulating data in epidemiology and genetics indicates a direct link between the circadian clock and cancer through circadian regulations of cell cycle checkpoints as well as metabolic pathways.

The importance of controlled cell proliferation is not only an issue in cancer but is core component in proper tissue reconstitution after wound healing and shall be discussed in detail in chapter 3.2 the paper “NONO couples the circadian clock to the cell cycle” (submitted) showing that hyperproliferation in dermal cells as well as loss of cell cycle gating by the circadian clock abolished normal wound healing. This was the case in both, NONO-depleted as well as circadian clock deficient mice. Therefore NONO acts as a linker between circadian clock and cell clycle.

Chapter 2 – Aims

The multifunction nuclear protein NONO is found in a variety of complexes, such as RNA transport granules in neurons (Kanai et al. 2004), members of RNA-rich nuclear domains called paraspeckles (Fox et al. 2005, Prasanth et al. 2005), splicing complexes (Liang and Lutz 2006), and transcriptional repression complexes (Wu et al. 2006). Because we have found NONO to interact with the circadian clock protein PER1 (Brown et al. 2005b), the goal of this thesis was to understand how NONO might be important for mammalian circadian clocks and their control of physiology.

Therefore a *Nono*-gene trap (*Nono*^{gt}) mice strain was made to characterize the functions of this protein in the circadian clock. An integrative approach was used for phenotypic characterization of *Nono*^{gt} mice on an organismic level, and subsequent gene expression studies compared wildtype and *Nono*^{gt} mice as well as their cells using *in vitro* model systems.

2.1 Circadian clocks in embryonic stem cells

Preliminary results implicating NONO in the circadian clockwork were obtained using an embryonic stem (ES) cell line gene-trapped for NONO (Bay Genomics clone ID: YHA266). In addition to the results about NONO described below, while working with ES cells it became clear that undifferentiated ES cells do not possess a functional circadian clock. Since the question of circadian ontogeny is little understood, we decided to explore this subject by controlled differentiation of ES cells.

We developed an improved ES-differentiation protocol to get neurons from ES cells. Circadian rhythms were monitored overall several days by infecting these cells with an adenoviral vector designed for this purpose that contains a luciferase gene under the control of a clock gene promoter. Our subsequent experiments succeeded in identifying an approximate time-frame for circadian clock induction during development.

2.2 Characterization of NONO function in the circadian clock using a *Nono*^{gt} knockout mouse line

In the YHA266 ES cell line a genetrap cassette was inserted in the second intron of the *Nono* gene prior to its translational start, so that NONO protein was no longer made.The observation first made by Brown *et al.* that fibroblasts depleted of NONO by RNAi have shorter period length (Brown et al. 2005b) was confirmed in differentiated *Nono*^{gt}-neurons compared to differentiated wildtype neurons. A NONO-deficient mouse line was produced via blastocyst injection of ES cell line YHA266 resulting in the *Nono*^{gt} line.

A second goal of this thesis was to characterize circadian defects in these mice, using behavioral assays in running wheels, complemented with studies of gene expression and

protein levels monitored over a 24-hr time frame. For *in vitro* assays adult dermal fibroblasts (ADFs) extracted from tail were used. In addition to circadian phenotypes, we also observed hyperproliferation in *Nono*^{gt}-derived ADFs. These observations resulted in our investigation of senescence and wound healing phenotypes in these mice, and ultimately allowed us to study NONO-mediated coupling between the circadian clock and the cell cycle.

2.3 Characterization of NONO homologs and their contribution to circadian clockwork – *in vitro* and in genetrapped mouse lines

The moderate period shortening in *Nono*^{gt} mice was comparable to changes in other mice mutant for redundant core clock genes, i.e. genes whose function is duplicated by a homolog. Checking for NONO homologs by using the conserved domain architecture tool (CDART) from NCBI, several potential candidates for NONO homologs were identified. Among them were the splicing factor SFPQ and the paraspeckle component 1 (PSPC1) protein.

To support the hypothesis of a possible substitution of NONO function by one of these proteins, preliminary data was acquired in a cellular model system by modulating expression levels using vectors that either overexpress or repress one of the three proteins. Effects on both acute and long term response in the transcription of clock gene reporters was observed. Therefore mouse lines deficient for both NONO homologs were produced, and a final goal of this thesis became the characterization of circadian clock function in these mice and cells from them.

Chapter 3.1

The circadian clock starts ticking at a developmentally early stage (Paper 1)

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Abstract

Although overt diurnal rhythms of behavior do not begin until well after birth, molecular studies suggest that the circadian clock may begin much earlier at a cellular level: mouse embryonic fibroblasts, for example, already possess robust clocks. By multiple criteria, we found no circadian clock present in mouse embryonic stem cells. Nevertheless, upon their differentiation into neurons, circadian gene expression was observed. In the first steps along the pathway from embryonic stem (ES) cells to neurons, a neural precursor cell (NPC) line already showed robust circadian oscillations. Therefore, at a cellular level the circadian clock likely begins at the very earliest stages of mammalian development.

Introduction

Circadian rhythms of behavior in many animals are first visible weeks or months after birth. Nevertheless, a large body of evidence suggests that daily biological timekeeping could begin much earlier. For example, in Zebrafish the transcription of the clock gene *Per1* begins on the first day of development, and is already expressed in rhythmic fashion on the second day when fish are reared in a light-dark cycle (Dekens and Whitmore 2008). Even a brief pulse of light during the first day of development suffices to synchronize a circadian clock by day 3 (Ziv and Gothilf 2006). Since zebrafish cells are individually photoreceptive (Whitmore et al. 2000), it is thus likely that circadian environmental signals are immediately and directly transmitted to the developing embryo. In *Drosophila*, which also displays universal circadian photoreception (Plautz et al. 1997), a single pulse of light during the first larval stage immediately after hatching suffices to entrain a circadian rhythm of pupal eclosion several days later (Sehgal et al. 1992). Thus, here too a circadian clock is functional during the first day of development.

In mammals, when circadian timekeeping begins is unclear. For precocial species like humans, diurnal physiological rhythms like fetal heart rate, respiratory rate, movement, and plasma cortisol can be detected in utero. For altricial species like rats (whose pups are very immature at birth), these diurnal physiological signs are only visible postnatally. At a cellular and tissue level, though, circadian rhythms begin prenatally in both (reviewed in Davis and Reppert 2001, Seron-Ferre et al. 2001, Sumova et al. 2006).

The “master clock” of mammals is the suprachiasmatic nucleus (SCN) of the brain hypothalamus. In both classes of mammals mentioned above, circadian rhythms of physiology coincide roughly with the completion of neurogenesis in this nucleus and its innervation by the retinohypothalamic tract – by midgestation in precocial mammals, and much later in altricial ones (Seron-Ferre et al. 2001). Notwithstanding, rhythmic daily activity of this nucleus, measured both metabolically and by electrical activity in slice

culture, begins fetally in both groups (Reppert and Schwartz 1983; Shibata and Moore 1987). One of the most obvious consequences of these fetal clocks is that pups show identical phase to their mother, both *in utero* and postnatally (Davis and Gorski 1985). This synchrony is lost if the maternal SCN is ablated, so it is presumed that fetal rhythms are driven or entrained by maternal cues (Davis and Gorski 1988, Reppert and Schwartz 1986).

The mechanism of the circadian clock is cell-autonomous (Welsh et al. 1995) and present not only in the SCN, but in most cells of the body (Yamazaki et al. 2000). Its molecular mechanism is likely based upon interlocked feedback loops of transcription and translation of dedicated "clock gene" loci, including transcriptional activators like *Clock* and *Bmal1*, and transcriptional repressors like *Per1, Per2, Cry1, Cry2,* and *Rev-Erb*α (reviewed in Ripperger and Brown 2009). Direct measurement of clock gene transcripts, as well as the use of clock gene promoter-driven bioluminescent reporters (like *Per1-luciferase*), have permitted the dissection of fetal circadian oscillations at a gross scale. Between E10 and E21, expression of *Per1-luc* in fetal rats measured in one study *in vivo* steadily increases, with the first evidence of rhythmic expression occurring at E12 (Saxena et al. 2007). Nevertheless, specific analysis of SCN clock gene expression has failed to detect circadian oscillations in the early SCN in multiple cases (Li and Davis 2005, Sladek et al. 2004). Interestingly, although synchronous circadian oscillations cannot be detected in whole mouse embryos or tissues *in vivo*, they can be detected in some of the same tissues *ex vivo*, suggesting that a coherent synchronizing signal might be lacking (Dolatshad et al. 2010). Another recent study found rhythmic fetal clock gene expression in the *pars tuberalis* but not in the SCN, implying that early synchronous circadian oscillations might be the result of maternal signals such as the circadian hormone melatonin to input-driven organs (Ansari et al. 2009).

At a cellular level, the picture is a bit clearer. Although clock transcripts are present in the oocyte, their levels decrease steadily until the 16-cell stage, before rising again at the blastocyst stage (Ko et al. 2000). Multiple studies show oscillations of clock or clock reporter gene expression in explanted embryonic fibroblasts, so a cellular clock probably exists as early as E12 (Yagita et al. 2001). Nevertheless, it is unlikely that clocks are present in the earliest embryonic cells: while these studies were underway, work from Yagita *et al.* showed that embryonic stem cells do not possess functional circadian oscillators, though cells differentiated from them do (Yagita et al. 2010). These data suggest that a clock is absent in early pluripotential cell types and rapidly emerges as differentiation begins, even if synchronous circadian oscillations within tissues do not occur until later.

To test this hypothesis explicitly, we investigated circadian oscillations in an embryonic stem (ES) cell line, as well as in a neural precursor cell (NPC) line, and in neurons differentiated from both. Our results suggest that while the circadian clock is indeed silent in primordial stem cells, it is immediately activated in some of the earliest multipotential cells derived from it. Thus, the circadian oscillator "begins to tick" at a cellular level at the very earliest stages of mammalian development.

Results

Embryonic stem cells do not have a functional circadian clock

To investigate the circadian clock of mouse ES cells, we developed an adenovirus-based circadian luciferase reporter based upon the promoter of the *Bmal1* gene that was capable of infecting these cells at high titer without affecting their differentiation. After infection, cellular circadian rhythms in these cultures were synchronized with dexamethasone, and bioluminescence was measured during the next four days. Cosinor analysis demonstrated no significant rhythmicity (Fig 1A, top panel), whereas equivalent treatment of 3T3 fibroblast cells showed robust diurnal oscillations of reporter expression (Fig 1A, bottom panel). Equivalent results were obtained using synchronization with a simple medium change containing fresh serum (Fig 1A, arrow).

From the assays above, it is impossible to determine if the circadian clock as a whole is defective in these cells, or if only circadian transcription of our reporter gene is defective – e.g. due to lack of a critical promoter-binding factor. We tested this possibility by collecting RNA from dexamethasone-synchronized cultures at regular intervals over 24 hours and analyzing expression levels of a variety of clock genes. No rhythmic oscillation was observed in any gene. Compared to 3T3 cells, expression of some genes (e.g. *Bmal1, Per1, Per2*) was up to 1000x lower in ES cells, while others were 100x overexpressed (e.g. *Cry1*) or comparably expressed (e.g. *Cry2, Rev-Erbα*) (Fig 1B, Fig S1). We concluded that the existence of a functional circadian oscillator analogous to that in adult mammals is unlikely.

Both of the previous methods rely upon populations of cells. Hence, it is formally possible that individual ES cells demonstrate circadian gene expression that is unsynchronized to that of its neighbors. To rule out this hypothesis, we analyzed these cells via low-light microscopy to detect oscillations of bioluminescence in individual cells. No rhythmicity was observed (Fig 1C, Supplementary Movie 1).

Differentiated ES cells show normal circadian oscillations

Although we saw no circadian oscillations in ES cells, we nevertheless expected that circadian oscillations would occur in differentiated tissues. We therefore differentiated our ES cell cultures to attempt to restore circadian oscillations. Using cell aggregation and retinoic acid treatment, we were able to differentiate our ES cells homogenously to neurons, as evidenced by their clear dendritic and axonal outgrowths (Fig 2A,B). Upon synchronization with dexamethasone, these cells showed robust circadian oscillations of *Bmal1-luc* expression (Fig 2C) of amplitude equivalent to those in 3T3 cells (Fig 1A, bottom panel).

The activation of the circadian clock is developmentally early

One of the major problems in determining exactly when the circadian clock is activated is that the initial steps of differentiation occur relatively rapidly. For example, in mice it is clear from numerous publications that embryonic fibroblasts (MEFs) contain a functional circadian oscillator (Yagita et al. 2001), and these cells appear as early as embryonic day 12 (Strutz et al. 1995). Similarly, neurogenesis in most brain regions starts around day 9, and peaks at day 10-13 (Finlay and Darlington 1995). After the initial formation of endoderm and ectoderm from completely pluripotential stem cells, the first step in the differentiation of this lineage is the formation of neural precursor cells (NPCs), a heterogenous population of cells in the embryonic ventricular zone which still divide vigorously but can subsequently differentiate into neurons or astrocytes (Gotz and Sommer 2005). Various cell line models exist for these NPC cells, all sharing the characteristics of neurosphere formation and expression of primordial markers like SOX2, Nestin, DLL3, HES6, NOTCH4, and CD133. Using the cell line GS-5 that expresses all of these (Gunther et al. 2008), we cultivated these cells both as actively dividing neurospheres (believed to represent active NPCs (Svendsen et al. 1998)) (Fig 3A) and, after treatment with retinoic acid, nondividing and differentiated into neurons (Fig 3B). Both cultures were infected with reporter virus, synchronized as above, and measured via real-time luminometry. Identical robust rhythms were observed in both cells (Fig 3C, D, Fig S2), implying that the circadian clock already exists at the NPC level. Equivalent results were seen with another line (GS-8, data not shown), but its culture resulted in far greater heterogeneity of cell morphologies.

Discussion

In this paper, we show that the activation of the circadian oscillator occurs during one of the earliest steps of development, at least in the lineage that we examined. Although an embryonic stem cell line showed no functional circadian clocks, a neural precursor cell

line and neurons differentiated from both lines showed robust clock activity. Nevertheless, overt rhythms of behavior do not manifest themselves prior to a few weeks of age in mice, and two months of age in humans (reviewed in Davis and Reppert 2001, Seron-Ferre et al. 2001). Hence, these cellular rhythms must be desynchronized or suppressed.

One *caveat* to the interpretation of our results is that we employed stable pluripotential cell lines. These lines may not be identical to the primary cells that they are designed to represent, and they are maintained in culture via a specialized mix of growth factors that prevent them from differentiating. It is possible that these specialized conditions are responsible for the suppression of an otherwise functional circadian clock. We consider this hypothesis unlikely because the same cocktail of growth factors was used to cultivate NPCs (which had clocks) and ES cells (which did not).

Secondly, whether a given cell line represents adequately the same population of cells *in vivo* is always a valid criticism. For ES cells, in answer we can say that these cells were subsequently used to generate mice in the course of a different study, verifying their pluripotency. For NPCs, the answer is less clear. Various NPC cell models all suffer from the fact that they are isolated from brain tumors (Svendsen et al. 1998, Tarnok et al. 2010). The principal evidence of their validity as a model system is their expression of primordial cell markers and their ability to be differentiated. We chose the GS-5 line because we could maintain it in culture without traces of differentiated neurons under one set of conditions and differentiate it completely with another, and because it expresses numerous primordial markers (Gunther et al. 2008).

The fact that circadian oscillations are not observed in ES cells, but can be observed in cells differentiated from them, is confirmed by similar work from Yagita *et al.* In their study, the proof that ES cells *per se* possess no normal functional circadian clock is elegantly shown by an additional reverse experiment: de-differentiation of clock-containing cells back to ES cells results in the elimination of functional circadian oscillations (Yagita et al. 2010). They also show that NPCs possess functional circadian clocks; however, since their NPCs are partially differentiated from their ES cells, the media for these types contain different cocktails of growth factors. Hence, it is possible that the differences that they observe could be driven by external factors. Our own work uses identical media for both cell types to eliminate this possibility, but instead has the limitation that our NPC cells are cancer-derived. The two studies together, by examining different ES and NPC lines cultivated under different conditions but assayed by essentially identical methods, make it highly probable that the essential conclusions of both studies are correct.

In normal development, a completely pluripotential ES cell would linger less than a day and a multipotential NPC only 3-5 days, making the question of their circadian clock

rather academic. More interesting is the question of why a circadian clock is activated so early at a cellular level compared to a behavioral or physiological one. The viability of knockout mice with defective circadian clocks suggests that circadian rhythms in gene expression play no essential role during development. Hence, very early cellular activation of the circadian clock might occur by chance at the same time as other cellular processes, but before the systemic cues necessary for its synchrony and organism-wide manifestation. A second possibility, which we find more interesting, is that a cellular clock itself provides a valuable function even in the absence of intercellular synchrony by segregating mutually harmful processes such as respiration and cell division, and thereby conferring a selective evolutionary advantage – e.g. more effective DNA repair (Collis and Boulton 2007, Sahar and Sassone-Corsi 2009).

In any case, it is clear from our results that the circadian clock occurs at a very early time in development. How it is switched on is probably related to the appearance of a full complement of gene products necessary to circadian function, in the ratios necessary for a limit cycle of feedback. *Why* it is switched on is a question that shall only be answered by much further experimentation.

Figure 1

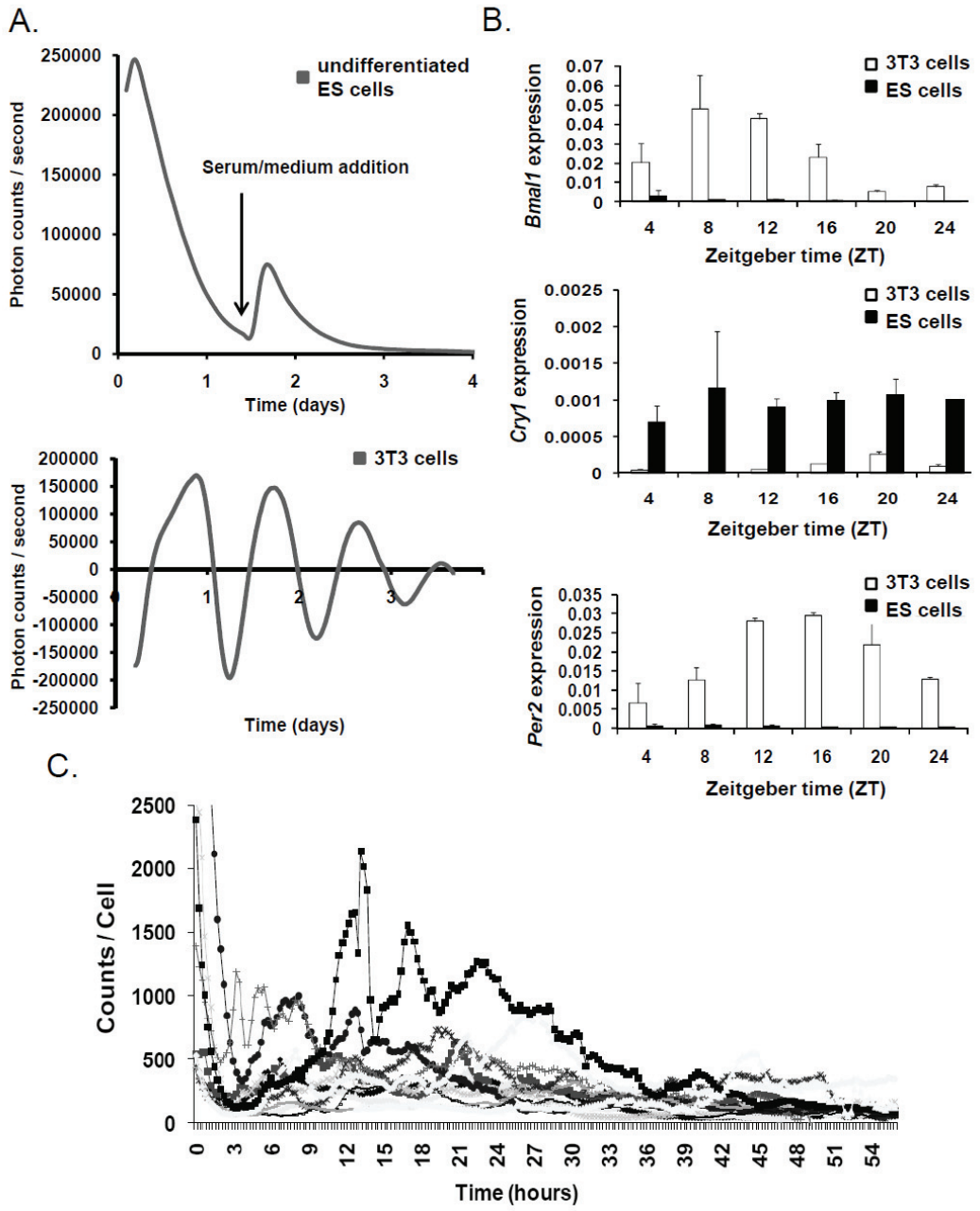


Figure 1

A. Top panel, bioluminescence from undifferentiated ES cells treated with dexamethasone to synchronize putative circadian oscillations. Arrow, a second synchronization attempt of the same culture using fresh medium + 20% serum. Y axis, bioluminescence (photon counts per minute). X axis, time in days relative to dexamethasone treatment. Bottom panel, bioluminescence from 3T3 fibroblast cells treated with dexamethasone to synchronize putative circadian oscillations. Y axis, deternded bioluminescene (absolute photon counts per minute relative to 24-hour average of bioluminescene levels); X axis as in A. **B.** Expression of *Bmal1*, *Cry1,* and *Per2* gene expression by qPCR from identical plates of undifferentiated ES cells synchronized as in A. In comparison, expression of the same genes in identically synchronized 3T3 fibroblast cells is also shown. X axis, hours after synchronization. Y axis, gene expression expressed as a ratio relative to GAPDH. **C.** Bioluminescence traces of individual cells from undifferentiated ES cell cultures synchronized as in A. Y axis, pixel intensity per cell.

Figure 2

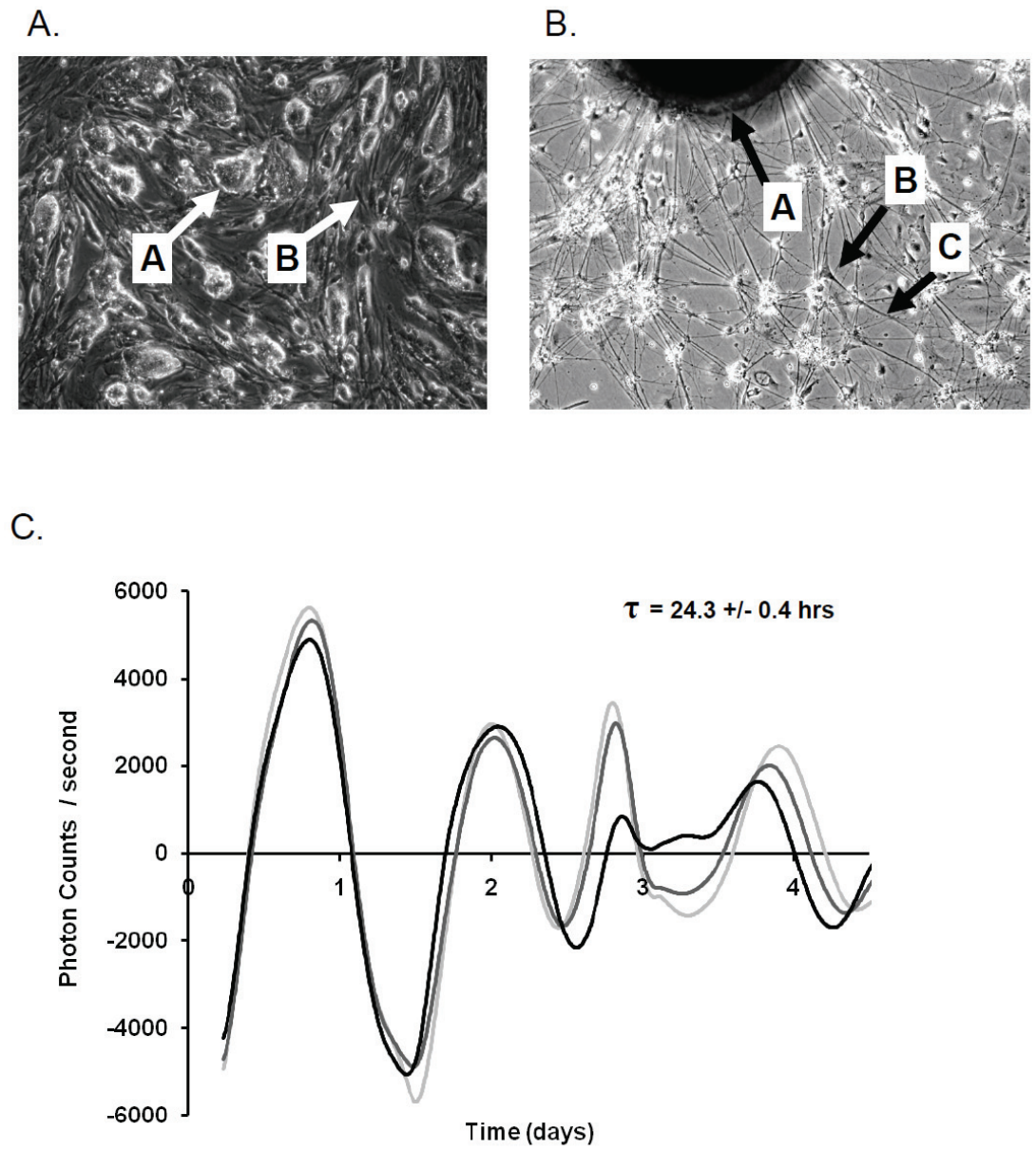


Figure 2
A. Undifferentiated ES cells, grown on a feeder layer of mitotically inactivated fibroblasts. Arrow A, ES cell colony. Arrow B, fibroblast. **B.** Neurons derived from differentiated ES cells. Arrow A, remains of embryoid body. Arrow B, neuron. Arrow C, axon **C.** Bioluminescence from cells in B. X axis, time in days relative to dexamethasone synchronization; Y axis, relative bioluminescence (photon counts per second, detrended). Tau is calculated as the average from three independent experiments. Traces are shown from technical triplicates of a single experiment.

Figure 3

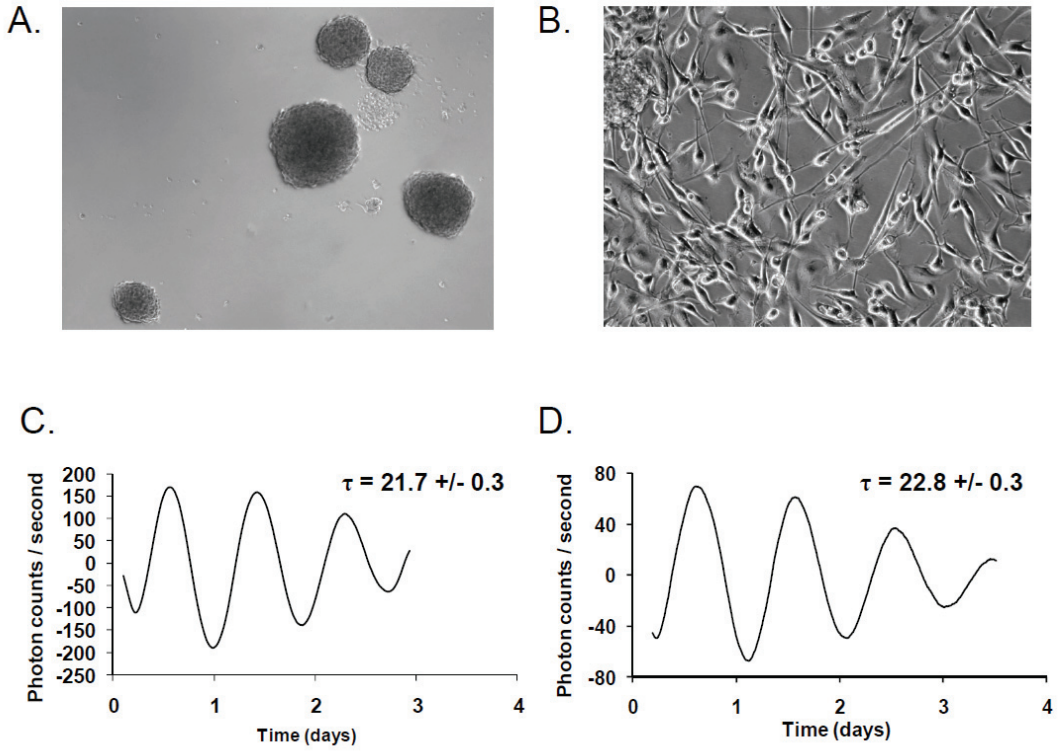


Figure 3
A. Undifferentiated GS-5 cells, grown as neurospheres. **B.** Neurons derived from GS-5 cells. **C.** Bioluminescence from cells in A. Y axis, relative bioluminescence (photon counts per second, detrended. X axis, time in days relative to dexamethasone synchronization. **D.** Bioluminescence from cells in B. Tau is calculated from three independent experiments. Traces are shown from a representative culture.

Supplementary Figure 1

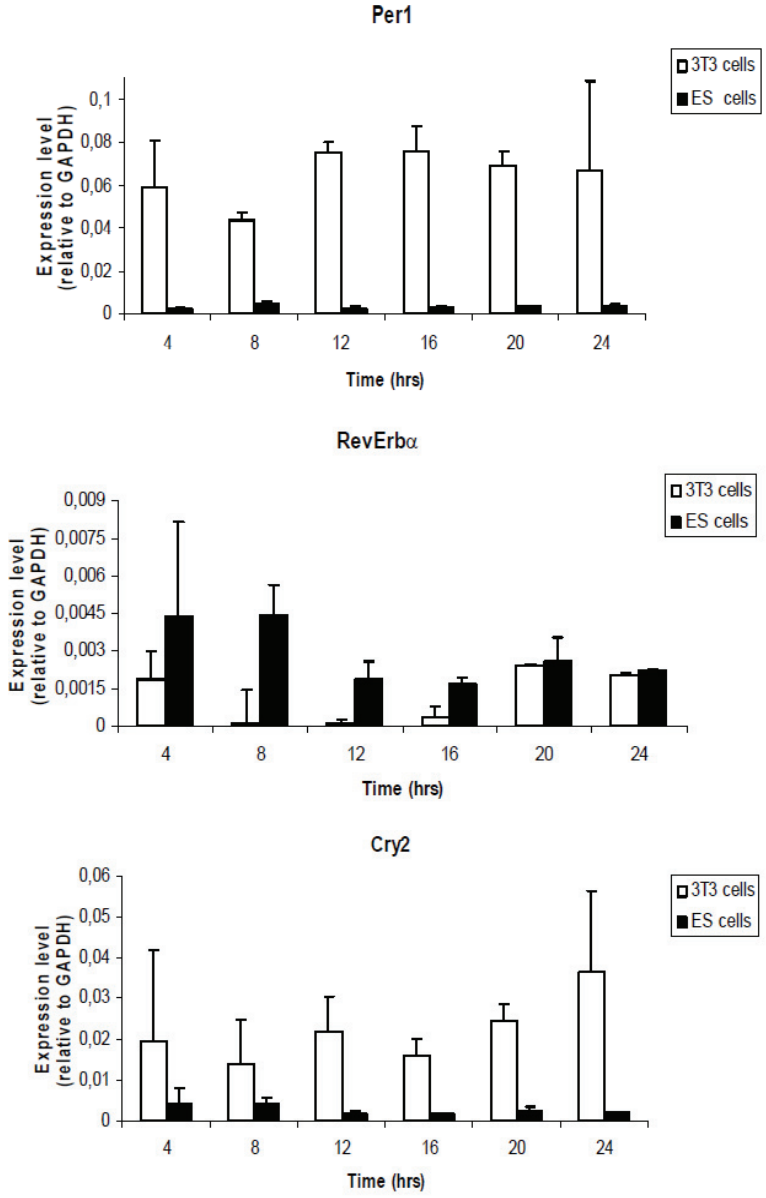
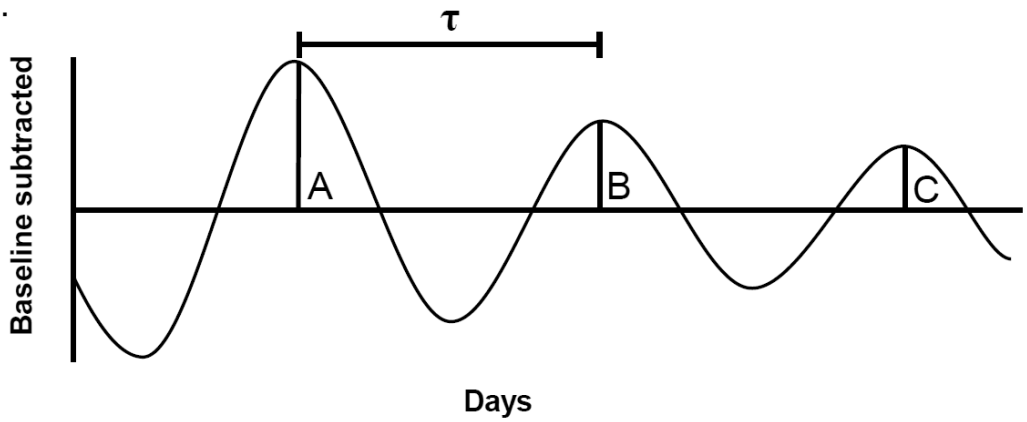


Figure S1
Expression of *Per1, Rev-Erba,* and *Cry2* gene expression by qPCR from identical plates of undifferentiated ES cells, compared to 3T3 fibroblasts. X axis, hours after synchronization with dexamethasone. Y axis, gene expression expressed as a ratio relative to GAPDH.

Supplementary Figure 2

A.



B.

Cell type	τ	A	Raw counts/sec	A/raw counts	B/A	C/A
3T3	23.9	200000	1000000	0.20	0.82	0.47
	23.6	160000	1100000	0.15	0.89	0.56
GS-5 undiff.	22.4	120	1100	0.11	0.60	0.35
	21.0	160	1400	0.11	0.90	0.65
GS-5 diff.	23.0	75	600	0.13	0.85	0.50
	23.3	225	900	0.25	0.75	0.35
ES cell diff.	25.1	6000	30000	0.20	0.50	0.40
	24.7	5500	30000	0.18	0.50	0.35

Figure S2
A. Schematic diagram of parameters used to calculate relative amplitudes of circadian oscillation. **B.** Relative amplitudes of circadian oscillations of the different cell types examined in this paper: 3T3, neuronally differentiated ES cells, undifferentiated GS-5 NPCs, neuronally differentiated GS-5. Highly different raw counts/sec reflect different levels of lentiviral infection. A/raw counts provides a measure of the amplitude of circadian oscillation. B/A and C/A provide a measure of dampening.

Supplementary Movie.
Time-lapse movie showing luciferase expression of undifferentiated ES cells over 4 days. Quantification of individual cells from this recording was used to generate the graphs in Figure 1. http://jbr.sagepub.com/content/suppl/2010/12/08/25.6.442.DC1

Material and Methods

Culture and differentiation of embryonic stem cells
Wildtype mouse embryonic stem cells line E14Tg2A.4 came from Bay Genomics (UC Davis, US). Culture medium for embryonic stem cells consisted of KO-DMEM (Invitrogen) supplied with 15% FBS Gold (GIBCO), 2mM L-glutamine (GIBCO), 0.1mM non-essential amino acids, 1U/ml LIF (ESGRO) and 0.1mM β-Mercaptoethanol (Sigma). Cells were grown on a feeder layer consisting of mitotically incompetent mouse embryonic fibroblasts. Differentiation was achieved by hanging droplet culture of embryoid bodies (EBs, see Conley et al. 2005). After four days, individual EBs were differentiated for an additional four days in suspension culture in DMEM medium with 4500g/L glucose (Sigma) containing 10% FBS, 2mM L-gluthamine, 0.1mM non-essential amino acids, 0.1mM β-Mercaptoethanol and 5µM retinoic acid (Sigma). Differentiated embryoid bodies were collected by centrifugation and plated in neurobasal medium (GIBCO) supplemented with 0.5mM L-glutamine (GIBCO) and 1x B27^{TM} supplement (GIBCO TM, Cat. No. 17504-044)) on adhesive dishes coated with poly-L-lysine. After four to eight days the embryoid bodies flattened out, and cells had differentiated to neurons and formed a network on the plate.

Cultivation and differentiation of neural progenitor cell (NPC) lines
NPC lines GS-5 and GS-8 derived from human glioblastoma (Gunther et al. 2008) were cultivated in serum-free Neurobasal medium (GIBCO) supplemented with 2% B27^{TM} supplement (GIBCO TM, Cat. No. 17504-044), 2mM Glutamine (GIBCO), 20ng/ml human recombinant fibroblast growth factor-2 (PeproTech), 20ng/ml human recombinant epidermal growth factor (PeproTech) and 32IU/ml Heparin (Sigma). The cultures were incubated at 37°C with 5% CO_2. Under these culture conditions the cells grow as free-floating neurospheres. Once weekly the cells were split by gentle accutase^{TM} (Sigma, Cat. No. A6964) treatment followed by mechanical dissociation and washing to remove enzyme. The undifferentiated state was maintained by replenishing the growth factors every two days. For the differentiation, the neurospheres were collected by sedimentation, washed twice and plated as neurospheres in 35-mm polylysine-coated dishes in plating medium: Neurobasal medium supplemented with 2% B27^{TM} supplement (GIBCO TM, Cat. No. 17504-044), 2mM glutamine (GIBCO), 100U/ml penicillin/streptomicin (GIBCO), 10% foetal calf serum (Biochrom) and 1µM retinoic acid (Sigma). This plating favours neurogenesis over gliogenesis (Capowski et al. 2007). The cells were allowed to differentiate for 14 days, with medium change every 3-4 days.

Measurement of circadian clock properties in NPC lines
Infection with lentiviral reporter vectors was carried out as previously described (Brown et al. 2005) with a MOI of 10 at a confluency of 60% for undifferentiated and differentiated neurospheres. 500,000 cells in single cell suspension were seeded in a 12-well plate with 1 ml of concentrated virus. After 24- hours the cells appeared aggregated in neurospheres. The virus was removed by centrifugation and the neurospheres were seeded in 35-mm dishes with neurobasal medium containing B27 supplement, growth factors and heparin to keep them undifferentiated. The differentiation of infected neurospheres was done as described above. Fourteen days after infection, circadian rhythms of differentiated cells and neurospheres were synchronized with dexamethasone 400 nM for 20 minutes. After washing, medium without phenol red was supplemented with 0.1 mM luciferin and circadian rhythms were measured by real-time luminometry (Brown et al. 2005).
For neurospheres the growth factors were present during measurement.

Measurement of circadian clock properties in ES cell lines
Embryonic stem (ES) cells and the neurons differentiated from them as well as 3T3 cells were infected with recombinant adenovirus (vector from Invitrogen) containing a *Bmal1-luciferase* cassette identical to our previously-described lentiviral construct (Brown et al. 2008) with a MOI of 70 (for 293T cells) for undifferentiated ES cells at 70% confluency as well as 3T3 cells; and a MOI of 30 for neurons differentiated from ES cells at 40-60% confluency. We estimate that infection efficiency was in the range of 30-40%. To measure circadian bioluminescence, infected cells were synchronized with 100nM dexamethasone for 30 minutes. Thereafter the medium was changed to either complete embryonic stem cell medium as described above but supplemented with 0.1mM luciferin (for ES cells), or the complete medium for stem cell differentiated neurons supplemented with 0.1mM luciferin. Real-time bioluminescence was measured in a homemade photomultiplier-incubator apparatus at 37°C 5% CO_2 (Brown et al. 2005)

Clock gene expression analysis
RNA was extracted as described in *Current Protocols in Molecular Biology* (Kingston et al. 2001). 500ng of total RNA was transcribed to cDNA with SuperScript II (Invitrogen) using oligo(dT) primers according to manufacturer's instructions. For quantitative real-time PCR 20ng of cDNA was used and transcript levels of genes were detected by Taqman probes used with the Taqman PCR mix protocol (Roche) using the AB7900 thermocycler as described previously (Preitner et al. 2002).

Bioluminescence time-lapse microscopy and data analysis
ES cells were plated in 35-mm glass bottom dishes (Willco-dish, type 3522, Willco Wells B.V.) covered with laminin. After stimulating the cells with 100 nM dexamethasone for 30 minutes, the medium was replaced by 2 ml phenol red-free DMEM supplemented with 10% FCS and 1 mM luciferin. Bioluminescence imaging was performed on an Olympus LV 200 bioluminescence workstation equipped with a 20x UPLSAPO objective (NA 0.75). Cells were kept in a 37°C chamber equilibrated with humidified air containing 5% CO_2 throughout the microscopy. Bioluminescence emission was detected for several consecutive days using an EM CCD camera (Image EM C9100-13, Hamamatsu, Japan) cooled to -90°C using exposure times of 30 min. The image series were analyzed employing the ImageJ 1.32 software (National Institutes of Health, Bethesda, MD, USA; as described in Supplementary materials). To measure the varying bioluminescence response across image sequences from moving cells, we used a slightly adapted version of SpotTracker (Sage et al. 2005), an ImageJ plugin developed by us, as described previously (Dibner et al. 2009).

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Author Contributions

E.K. conducted the embryonic stem cell differentiation, bioluminescence recordings from undifferentiated and differentiated ES cells and analyzed the data. E.M. conducted the GS-5 cell differentiation, bioluminescence recordings from undifferentiated and differentiated GS-5 cells as well as the qRT-PCR and analyzed the data. C.D. and C.B. performed bioluminescence time-lapse microscopy and analysis of data. **E.K.** designed figures. S.A.B. and **E.K.** wrote the paper. All authors made comments on the manuscript.

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Chapter 3.2

NONO couples the circadian clock to the cell cycle (Paper 2)

Revision of this paper was resubmitted as:

NONO couples the circadian clock to the cell cycle.

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Abstract

Mammalian circadian clocks restrict cell proliferation to defined time windows, but this process is not well understood. NONO was previously found to interact with the circadian clock PER proteins. We now show that fibroblasts from NONO-deficient mice displayed an elevated cell doubling rate and a lower rate of cellular senescence. Both of these phenotypes were based on a loss of circadian activation by NONO of the *p16-Ink4A* gene. Lack of either NONO or canonical clock genes eliminated circadian cell cycle gating *in vitro,* and resulted in defective wound repair *in vivo*. Our results suggest that NONO couples the cell cycle to the circadian clock, and that this control may be useful to temporally segregate cell proliferation from tissue organization.

Introduction

The circadian clock adapts organisms to their daily surroundings both behaviorally and physiologically. In animals, not only are complex behaviors such as sleep and mood governed by this oscillator, but also different body functions such as digestion, circulation, and respiration (*1*). The basic mechanism of this clock is cell-autonomous in all studied species. In mammals individual clocks in most cells are synchronized by a brain “master clock” in the suprachiasmatic nucleus (SCN) of the hypothalamus in order to orchestrate all rhythmic physiology (*2*). On a cellular level, circadian physiology extends even to processes such as proliferation (*3, 4*), apoptosis (*5*), and DNA damage repair (*6*), which are thought to play important roles in cancer control (*5, 7*). Nevertheless, the exact mechanism of this regulation is at the moment little understood.

In individual cells, the circadian clock mechanism consists of oscillating feedback loops of transcription of “core” oscillator genes and posttranslational modifications of their protein products that regulate protein stability, activity, and/or localization. For example, in mammals the transcription of *Periods* (Per) and *Cryptochomes* (Cry) are activated by BMAL1:CLOCK heterodimers at *cis*-acting elements called E-boxes, and their protein products form complexes that repress their own transcription (*8*). We originally identified the RNA-binding protein NONO (also called p54nrb) biochemically as a new member of this circadian transcriptional repressor complex in mice, and mutation of its homolog *NonA* in flies resulted in severe attenuation of circadian rhythmicity (*9*). However, apart from its interaction with this circadian repressor complex, NONO’s mechanism of action within the clock remains unknown.

Results and Discussion

In order to better understand this issue, we used a "genetrapped" embryonic stem cell line (*10*) to generate *Nono*^{gt} mice that completely lack coding *Nono* transcript and NONO protein in all tissues tested (Fig 1A). In addition to a circadian phenotype of moderate behavioral period shortening that was expected from our previous studies (Kowalska *et al*, submitted), we noticed that the primary fibroblasts taken to characterize these mice showed more robust division than their wildtype counterparts. To quantify this effect, we serially split cells from wildtype (WT) and *Nono*^{gt} littermates at a predetermined ratio, and counted cells at each passage. *Nono*^{gt} cells indeed showed a markedly increased rate of population doubling relative to WT cells (Fig 1B). In principle, such an effect could have arisen either because cells divided faster or because they reached senescence – the normal postproliferative arrest of cell division in adult tissues – at a lower rate. To examine senescence, we stained the same cells for senescence-associated β-galactosidase Activity (*11*) at each passage from their initial isolation until their complete senescence. *Nono*^{gt} cells exhibited a roughly twofold decreased proportion of senescent cells at every passage (Fig 1C).

If *Nono*^{gt} cells had reduced senescence rather than an increased division rate, then fewer cells should remain non-dividing in cultures of equivalent age. We tested this hypothesis by staining dividing *Nono*^{gt} and WT cells from the same passage with the permanent cytoplasmic stain CFSE (carboxyfluorescein diacetate, succinimidyl ester) and then determining dye content by flow cytometry four days later. This dye remains trapped within the cells, but is diluted with each cytokinesis. Hence, it provides a quantitative analysis of the percentage of a cell population that has divided (*12*). After this experiment, whereas all *Nono*^{gt} cells had divided at least once, forty percent of the WT cells had not divided (Fig 1D). Reintroduction of NONO into primary *Nono*^{gt} fibroblasts via lentiviral transduction slowed division and increased senescence, and addition of NONO to WT cells slowed division even further (Fig 1E), confirming the role of NONO in restraining cell proliferation and pointing to a probable role for this protein in the cell cycle.

To identify the cell cycle stage at which NONO has a role, we fixed actively dividing fibroblasts from WT and *Nono*^{gt} animals and labeled them with propidium iodide, a fluorescent DNA-binding dye (*13*). Subsequent FACS analysis allowed us to quantify the proportion of cells in different stages of the cell cycle in each population. Our results demonstrated a twofold increase of cells in synthesis phase of the cell cycle when NONO was absent. NONO may thus act as a regulator of exit from the G1 phase (Fig 2A). To

identify cell cycle genes that are misregulated in the absence of NONO, we compared expression of cell cycle genes in *Nono*^{gt} cells and their WT counterparts using PCR-based microarrays, and found transcriptional misregulation of multiple cell cycle genes (Fig 2B, Table S1). Already knowing that NONO acted upon senescence and the exit from the G1 phase checkpoint, we chose the *p16-Ink4A* locus as a possible target meriting further investigation. Indeed, RNA abundance of *p16-Ink4A* was downregulated in *Nono*^{gt} cells (Fig 2B, left inset) as confirmed by quantitative RT-PCR. P16-INK4A has been implicated previously as a regulator of the mitogen-responsive retinoblastoma pathway, and is one of the key cellular components regulating senescence. It is known to repress the cyclin D dependent kinases 4 and 6 (cdk4 & cdk6), resulting in a G1 arrest that slows cell division and promotes senescence (*14, 15*).

Since p16-INK4A negatively regulates cell division and positively regulates senescence, its repression is consistent with the phenotype observed in *Nono*^{gt} fibroblasts. As a control, we also looked at key regulators of other pathways known to control senescence: the DNA damage-responsive p53 locus, and tankyrase, a downstream regulator of telomere length. No differences were observed in the level of tankyrase mRNA between wildtype and knockout cells, and p53 was two-fold lower, a direction inconsistent with the phenotype that we observe. In addition, for all known upstream regulators of *p16-Ink4A* – the *Ets1*, *Ets2*, and *Id1* loci – transcript levels in *Nono*^{gt} cells were unchanged or inconsistent with the observed downregulation of *p16-Ink4A* (Fig S1A-E), supporting our hypothesis that *p16-Ink4A* is a possible direct regulatory target of NONO.

To examine whether NONO could regulate the *p16-Ink4A* locus transcriptionally, we cotransfected 3T3 fibroblast cells with a vector expressing NONO and with a luciferase reporter for *p16-Ink4A* promoter activity. The addition of NONO resulted in a dosedependent 14-fold increase in luciferase signal compared to the levels obtained with the reporter alone (Fig 2C), suggesting that NONO functions as a transcriptional coactivator of the *p16-Ink4A* locus. By contrast, transcription of a reporter containing the CMV promoter driving expression of a hybrid *p16-Ink4A-luciferase* transcript including the entire 3'-untranslated region was unchanged (Fig S2). Therefore, the effect of NONO on the *p16-Ink4A* locus is likely on the transcriptional rather than the post-transcriptional level.

To investigate whether NONO also acts directly as a transcriptional co-activator of *p16-Ink4A in vivo*, we performed chromatin immunoprecipitation (ChIP) of endogenous NONO protein with the *p16-Ink4A* promoter. In murine liver NONO indeed binds to the *p16-Ink4A*

promoter region (Fig 3A), and moreover it does so in circadian fashion; but it does not bind to the promoter of the *Ets1* gene, an upstream regulator of *p16-Ink4A* (Fig S3A). If this binding were serving the function of transcriptional activation as we predict, then *p16-Ink4A* mRNA should be (i) circadian in its abundance and (ii) loss of NONO should lead to nonrhythmic and low *p16-Ink4A* transcript levels. This is exactly what we find (Fig 3B).

We have shown previously that overall NONO transcript and protein levels are constant throughout the day, but that one particular NONO complex (also containing the PER proteins of the circadian oscillator) shows circadian variations in abundance (*9*). Since the *p16-Ink4*A promoter was occupied by NONO in circadian fashion, we also tested to see if PER proteins bound there. ChIP analyses from mouse liver demonstrated that PER2 proteins are indeed present at the *p16-Ink4A* promoter with the same kinetics as NONO (Fig 3C). Consistent with this result, in $Per2^{Brdm1/Brdm1}$ mutant mice the binding of NONO to the *p16-Ink4A* promoter was phase-shifted to an earlier time and reduced in its abundance, and identical changes were seen in the magnitude and phase of *p16-Ink4A* transcription itself (Fig S3B and C). These results suggest that a PER-NONO complex is responsible for the effects of NONO upon the *p16-Ink4A* promoter. To test this hypothesis explicitly and determine whether PER proteins are required for NONO to activate *p16-Ink4A*, we compared the ability of NONO to activate transcription of a *p16-Ink4A* reporter in transient transfections into fibroblasts from wildtype and $Per1^{brdm1/brdm1}/Per2^{brdm1/brdm1}$ mice (hereafter designated $Per1/Per2^{mut}$), which lack functional PER1 and 2 proteins and circadian clocks (*16*). Transcriptional activation by BMAL1/CLOCK was achieved in both wildtype and $Per1/Per2^{mut}$ cells to the same extent (Fig 3D). Whereas activation by NONO was observed in wildtype cells, no activation was observed in mutant cells (Fig 3D), so PER proteins are required for NONO activity (or *vice-versa*).

Since p16-INK4A in its turn gates the cell cycle, we reasoned that NONO might be one of the unknown regulators that couple the circadian clock to cell division. To test this hypothesis, we synchronized circadian rhythms in duplicate plates of WT and $Nono^{gt}$ fibroblasts using dexamethasone (*17*). Subsequently, these plates were fixed at different times after synchronization, and then stained with propidium iodide and analysed by flow cytometry to quantify DNA content. WT cells showed marked circadian variations in cell division, with threefold variation in the proportion of cells in S phase at morning and evening (Fig 3E, left panel). In $Per1/Per2^{mut}$ cells, no such variations were observed (Fig 3E, middle panel). This lack of circadian gating was equally observed in $Nono^{gt}$ cells, which divided throughout the day at a level equal to the peak of WT cells – consistent with the fact that p16-INK4A negatively regulates cell division (Fig 3E, right panel). Thus the

NONO protein is necessary for diurnal cell cycle gating in these cells, and its absence likely leads to a disinhibition of the G1-S transition at specific circadian phases due to low levels of p16-INK4A (Fig 3E).

In spite of this dramatic phenotype in primary cells, *Nono*^{gt} mice develop normally. Hence, we reasoned that its importance for cell division is probably confined to adult animals. One situation in which cell division in adult animals plays an important role is during wound repair. To test whether NONO is required for normal wound repair, we wounded the skin of adult WT and *Nono*^{gt} mice, closed the resulting full-thickness incisional wound with Steristrips, and followed the wound healing process after 3, 7, 13, and 20 days by histological wound healing scores. Prior to wounding, skin structure appeared indistinguishable among WT and *Nono*^{gt} animals (Fig S4), and shortly after wounding, scab formation, inflammation, and angiogenesis were normal (Fig 4A, left). However, later after wounding, histological analysis revealed profound alterations in both dermal and epidermal regeneration. Wildtype wounds exhibited good reepithelialization and granulation tissue organization. Granulation tissue consisted of oval or spindle fibroblasts (dark red) embedded in a dense fascicular system of collagen fibers (grey; Fig 4A middle). The epidermal layer was well structured and gave, together with the well organized granulation tissue, rise to new skin that reestablished tissue integrity (Fig 4A right). In *Nono*^{gt} mice, immature granulation tissue was characterized by continued fibroblast proliferation, occupying most of the wound area (red, Fig 4A right) by day 20. These plumped and round–to-polyhedral fibroblasts were distributed within a loose matrix with hardly any collagen production. Similarly, the keratinocyte layer (stained in pink) was characterized by hyperproliferation and little epidermal organization (Fig 4A, middle, right). Thus, even as collagen-secreting fibroblasts hyperproliferated in wounded *Nono*^{gt} animals, collagen secretion was dramatically diminished (Fig 4B). The dividing cells could form only an immature dysfunctional epidermal layer resting on disorganized granulation tissue, which prevented healing (Fig 4B, Fig S4).

Since collagen is secreted by fibroblasts, the dramatic lack of collagen in wounds in *Nono*^{gt} animals is not what we expected to see in the fibroblast-hyperproliferated wounds of these animals. It might hint, though, at the underlying function of the gating of the cell cycle by NONO. We hypothesized that regulated circadian cell division might allow for organized cycles of division and tissue building, and thereby facilitate the organization of complex tissue structures. By contrast, when cell division occurs constantly and randomly, such organization never occurs, resulting in the under-epithelialization we have observed. To test this hypothesis, we also tested wound healing in two strains of mice lacking

functional circadian clocks: *Per1/Per2*^{mut} mice and *Bmal1*$^{-/-}$ mice. Both strains also showed defective wound healing. Consistent with the requirement of PER proteins for NONO activity at the *p16-Ink4a* promoter, the *Per1/Per2*^{mut} mouse essentially phenocopied the NONO mutant mouse, showing a thick layer of immature granulation tissue that was dominated by fibroblasts as well as polymorphonuclear cells and lymphocytes at day 6 with high evidence of hemorrhages, vascular congestion and necrosis. (Fig 4C,D). *Bmal1-/-* mice also showed severe wound healing defects, though this time marked by lack of epithelial coverage and a highly disorganized granulation tissue throughout the whole observation period. Most wounds in *Bmal1*$^{-/-}$ mice consisted mainly of an inflammatory fibrin clot with hardly any fibroblast or keratinocyte proliferation by day 6. (Fig 4C,D). The phenotypes of both mice strongly support the idea that circadian clocks might gate cell proliferation to promote correct tissue structure, and that NONO mediates this effect. Since dermal tissue morphology during development in all three strains of mice appears normal, it is likely that this function is either unnecessary or redundant earlier in development.

NONO was initially identified by its homology to splicing factors (*18*) and as a *Drosophila* factor involved in courtship songs (*19*). Since then, diverse studies have described it as a protein with pleiotropic functions mainly involved in RNA processing and transport, as well as a transcription factor (*20*). It has been implicated in diverse pathways such as nuclear receptor signaling (*21*), DNA repair (*22, 23*), and viral infection (*24*). Here, we have shown that it can directly activate the *p16-Ink4A* locus, an important regulator of the G1 exit checkpoint of the cell cycle. Thus, in adult fibroblasts NONO serves as the functional link between the circadian clock, senescence, and the cell cycle. The detailed mechanism of its coactivator function remains unclear. In the case of TORC-mediated coactivation by NONO, Amelio *et al.* suggested that NONO directly bridges interactions between RNA polymerase II and other activators (*25*). Other groups have shown that NONO regulates nuclear retention of RNAs (*26, 27*) or transcription termination (*28*), so it is possible that NONO couples transcription initiation and downstream RNA processing.

As more is known about the feedback loops that control circadian clock mechanism, increasing numbers of labs have begun to address the questions of how and why this oscillator communicates timing to the bewildering array of physiological and cellular process that it governs. Here, a pattern has begun to emerge: basic clock components can themselves directly regulate genes involved in the “output pathways” that control diurnal function (*29*). Within the cell cycle, it has been suggested that transcriptional

control of cell cycle genes such as *p21Waf1* (*30*) and *Wee1* (*3*) might play important roles, and that the *Per* genes themselves function as tumor suppressors (*31, 32*).

In this paper, we show that the NONO protein – which we have identified previously as an important component of the basic circadian oscillator in *Drosophila* and in mouse cells (*9*) – plays an equally important role to gate circadian cell division in fibroblasts. Importantlly, elimination of NONO entirely abrogated circadian-cell cycle coupling, suggesting that NONO serves as a necessary link between these two processes. This uncoupling allowed us for the first time to probe possible functions of this link. *Nono* mutants showed defective tissue structure during wound healing, and this defect was phenocopied in “canonical” circadian clock mutants. Therefore, we propose that circadian control of the cell cycle might be important for correct tissue structure during regeneration. More generally, co-opting the circadian clock as a synchronizing timekeeper – completely independent of its role in diurnal adaptation – might provide an important future paradigm for clock function at a cellular level.

Fig. 1

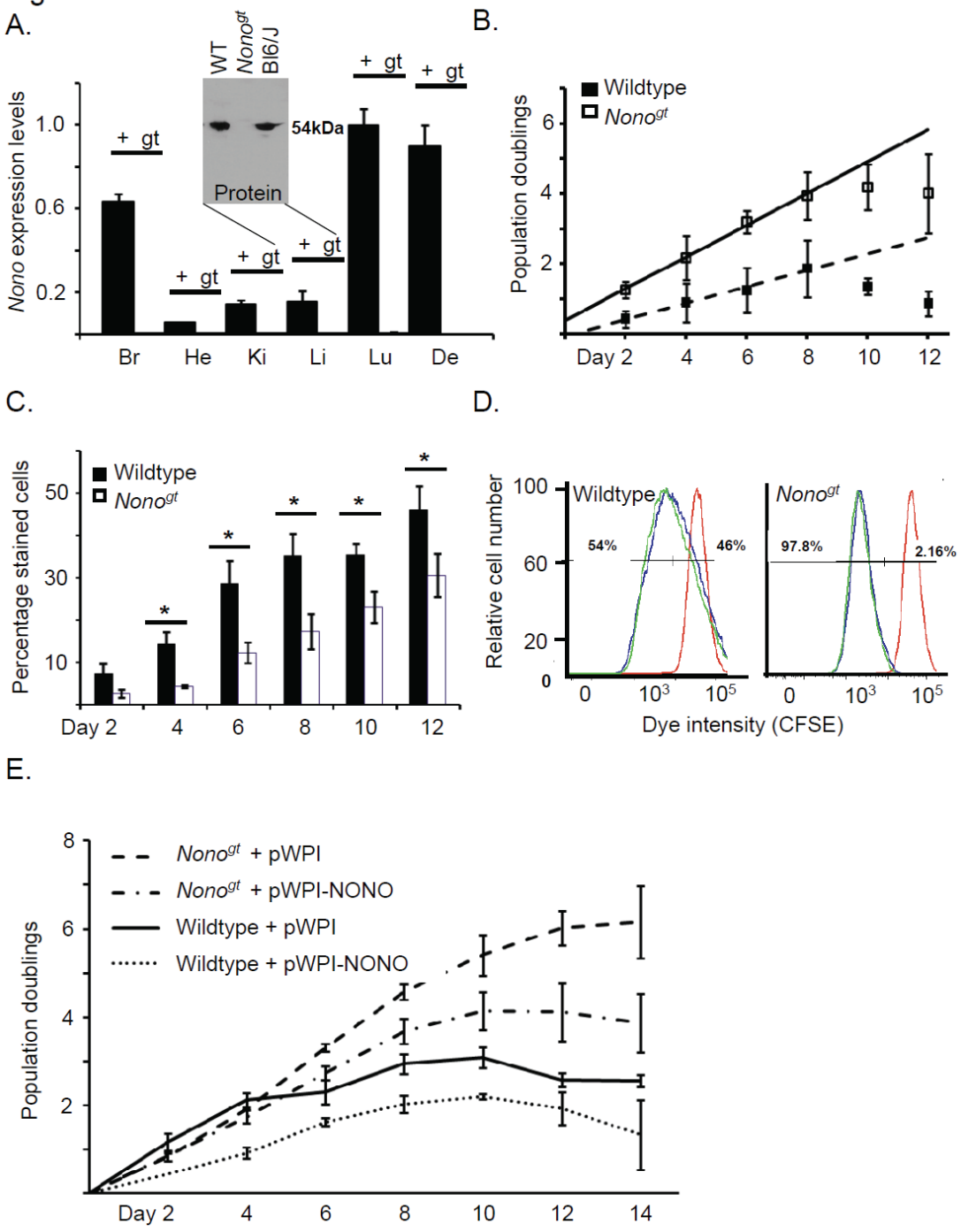

Fig. 1. (A) NONO RNA expression measured by qPCR in various tissues taken from WT (+) and *Nono*^{gt} animals (gt, not detectable). Y-axis, expression levels relative to maximum observed expression. Br=brain, He=heart, Ki=kidney, Li=liver, Lu=lung, De=dermis. **Inset**, NONO protein measured in liver nuclear extract from the same animals, as well as in unrelated C57-Bl6J mice (Bl6/J). **(B)** WT and *Nono*^{gt} primary fibroblasts were counted and passaged every two days, and a constant number of cells plated to a new dish. Total cell number over time is plotted relative to initial cell number as population doublings. (Student t-test for significant difference of doubling rates, p=0.05) **(C)** Cells from each passage in B were stained for SA-βgal activity, and the percentage of total cells expressing this marker were recorded. In this and all subsequent figures, * = p<0.05 and ** = p<0.01 (student ttest). **(D)** Duplicate non-confluent plates of WT and *Nono*^{gt} primary fibroblasts were stained with CFSE and allowed to divide for 4 additional days. Dye intensity was then measured by flow cytometry (duplicate plates of cells in green and blue). As a control, other plates of the same cells were treated with mitomycin C to inhibit cell division immediately after staining and then treated in parallel (red). Numbers near curves reflect the

percentage overlap between the green/blue curves and red curve. **(E)** WT and *Nono*^{gt} primary fibroblasts were infected with lentivirus WPI (expressing GFP) or WPI-NONO (expressing NONO), and allowed to proliferate via serial passaging as in A. Relative cell number for each cell type was plotted at each passage (i.e. every two days).

Fig. 2

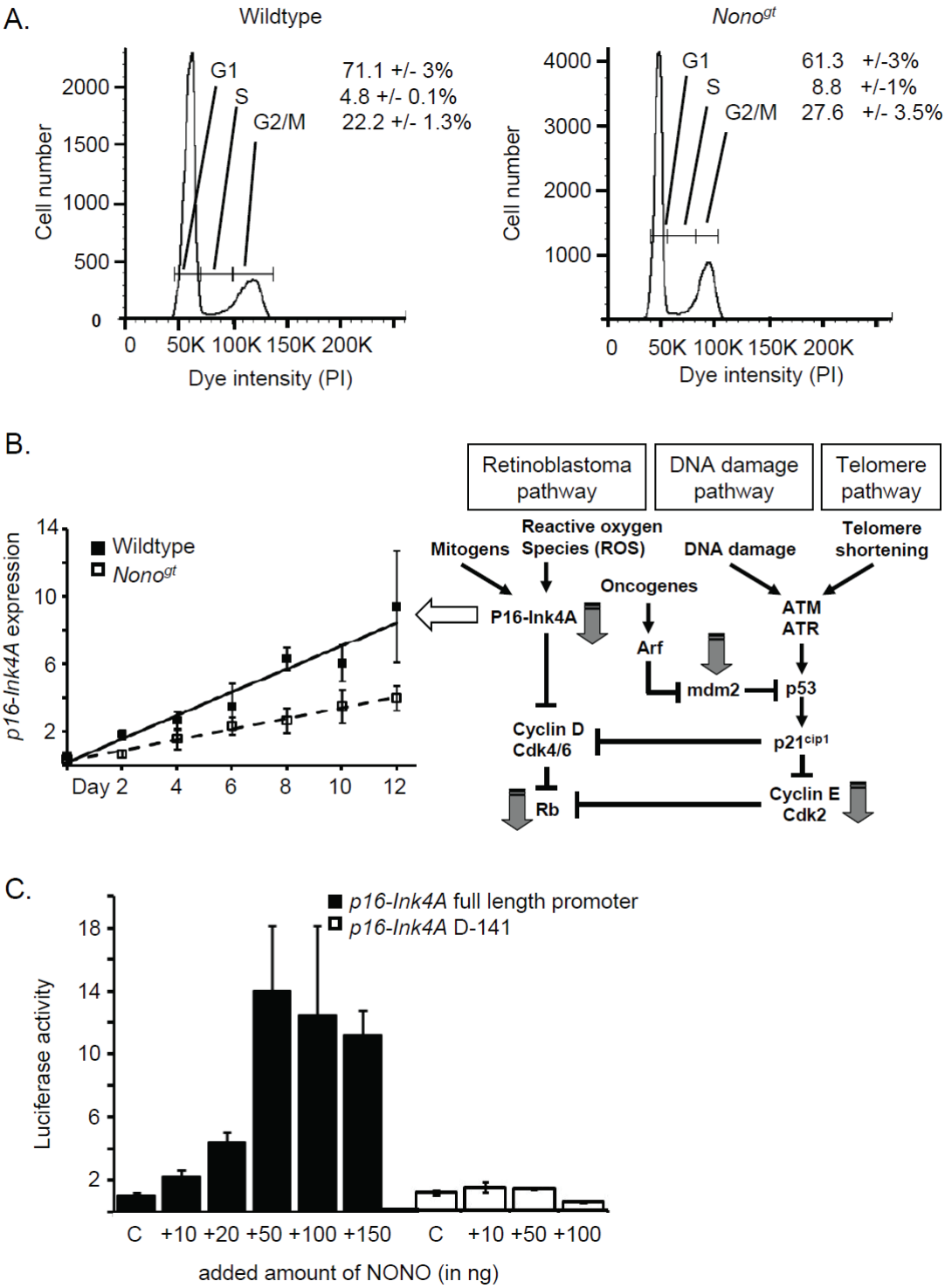

Fig. 2. **(A)** Dividing cultures of WT and *Nono*^{gt} primary fibroblasts were stained with propidium iodide, and analysed by flow cytometry to quantify cellular DNA content. **(B)** Total RNA was harvested from cells under conditions as in A, and subjected to qPCR array analysis. A selection of genes relative to senescence pathways are shown. Genes downregulated in *Nono*^{gt} cells are shown with grey arrows. **Inset**, Total RNA was harvested from each passage of WT (black squares) and *Nono*^{gt} (white squares) primary fibroblasts passaged serially as in Fig 1B. *p16-Ink4A* transcript levels were determined by qPCR for each passage. (Expression is shown relative to wildtype at Day 0, Student t-test for significant difference of *p16* accumulation rates, p=0.01. All array targets and their relative regulation are listed in **Table S1**. **(C)** 3T3 cells were transfected with a luciferase reporter plasmid driven by 0.8kb of the *p16-Ink4A* promoter (D-761), as well as

indicated amounts of a plasmid expressing NONO. After two days, relative bioluminescence in cellular extracts was determined (solid bars; data plotted in arbitrary units, signal from reporter alone = 1). Comparable experiments were performed using a reporter construct containing only a minimal *p16-Ink4A* promoter, 0.14kb (D-141) (open bars).

Fig. 3

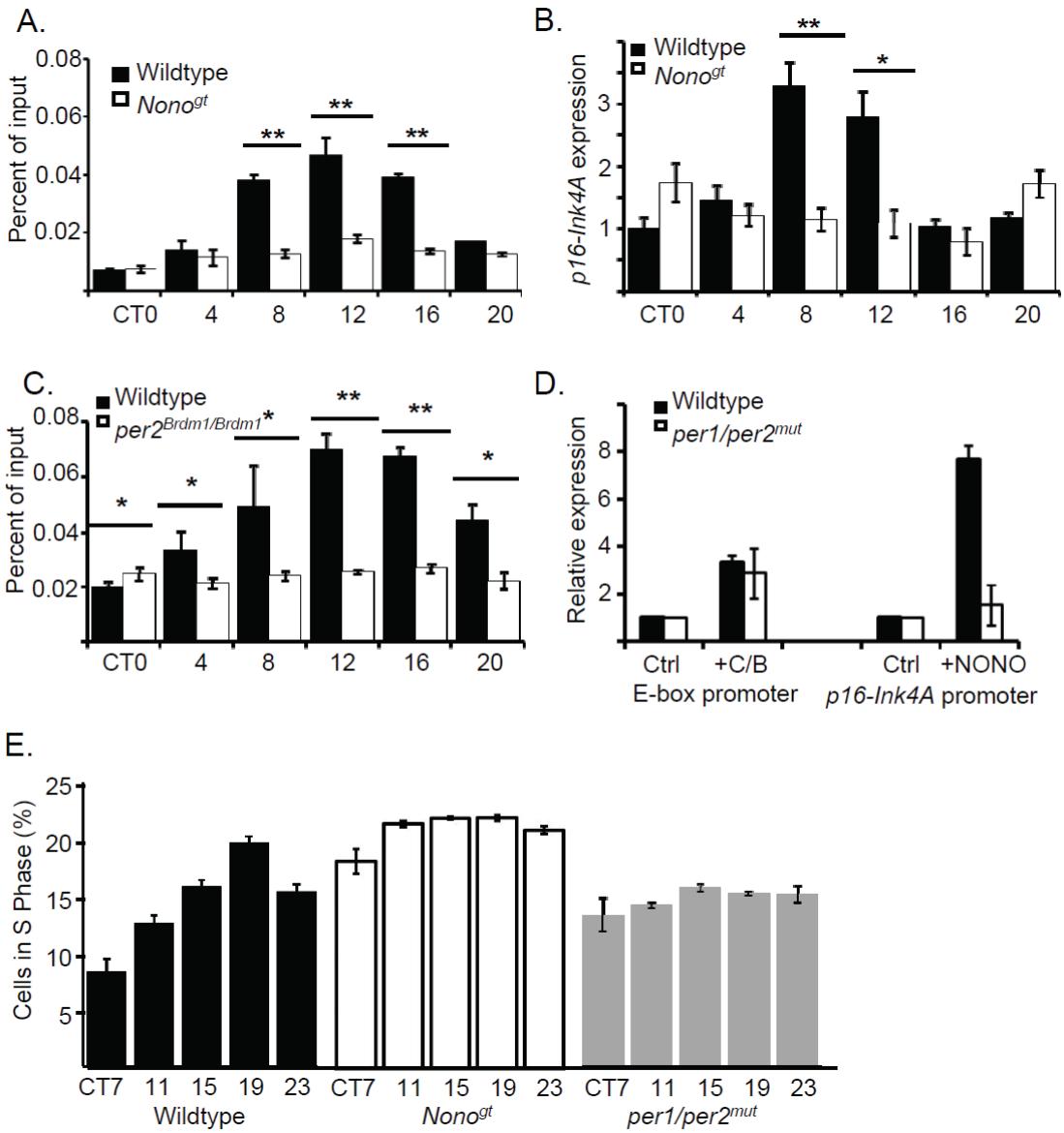

Fig. 3. (A) Liver chromatin was harvested from WT and *Nono*^{gt} mice at different times of day, and subject to immunoprecipitation using an anti-NONO antibody. Genomic DNA was purified from the precipitate, and DNA from the *p16-Ink4A* promoter was quantified by qPCR. **(B)** RNA was harvested from the livers in A, and *p16-Ink4A* transcript levels were quantified by qPCR. **(C)** ChIP analyses identical to those in (A) were conducted, but using chromatin harvested from WT and *Per2*$^{Brdm1/Brdm1}$ mice, and then precipitated with an anti-PER2 antibody. **(D)** Transfection experiments identical to those in Fig 2C were performed using primary fibroblasts isolated from wildtype mice and *Per1/Per2*^{mut} mice. **(E)** Dividing primary fibroblasts were clock-synchronized with dexamethasone, and then harvested at different times of day, fixed, and stained with propidium iodide. The percentage of dividing cells (i.e. in S phase) from WT (left panel), *Per1/Per2*^{mut} (middle panel), and *Nono*^{gt} cultures (right panel) was then quantified by FACS analysis. (Times indicated are relative to synchronization.)

Fig. 4

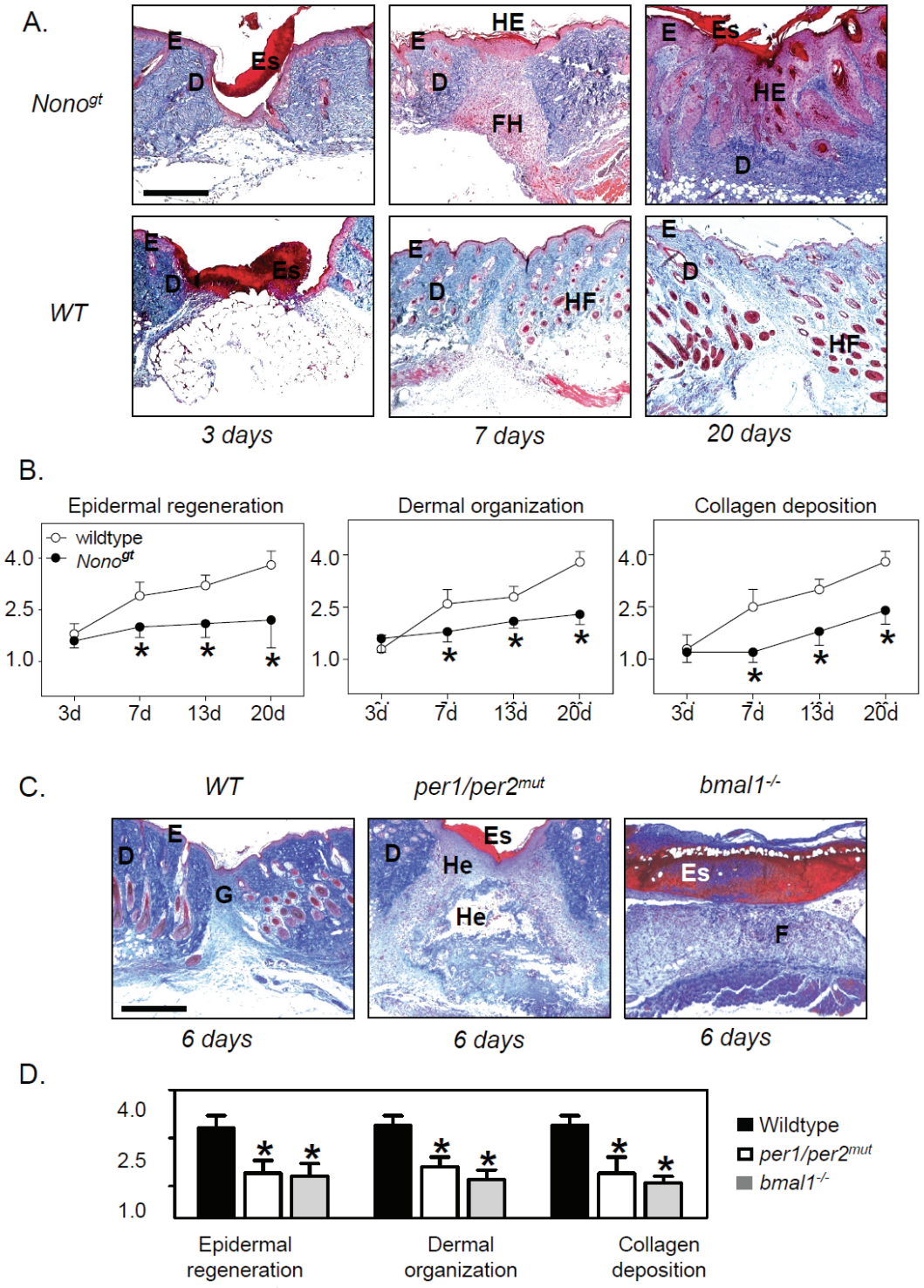

Fig. 4. **(A)** WT and *Nono*^{gt} mice were incisionally wounded, and then sacrificed 3, 7, or 20 days later. Representative Masson-Goldner trichrome-stained paraffin sections from the center of these wounds are shown. **(B)** Wound healing subscores of normal healing WT mice compared to *Nono*^{gt} mice on day 3, 7, 13, and 20 after incisional wounding in the dorsal skinfold. Data represent means ± SD (n=5). **(C)** Masson-Goldner trichrome staining of 6-day wounds from WT (left panel), *Per1/Per2*^{mut} (middle panel), and *Bmal1*$^{-/-}$ mice (right panel). D, dermis; E, epidermis; Es, Eschar; He, hemorrhaghe; HE, hyperproliferative epithelium; HF, hair follicle; F, fibrin; FH, fibroblast hyperproliferation; G, granulation tissue. Bar=1 mm. **(D)** Wound healing subscores of normal healing WT mice compared to *Per1/Per2*^{mut} and *Bmal1*$^{-/-}$ mice on day 6 after incisional wounding in the dorsal skinfold. Data represent means ± SD (n=6).

Supplementary Figures

Fig. S1

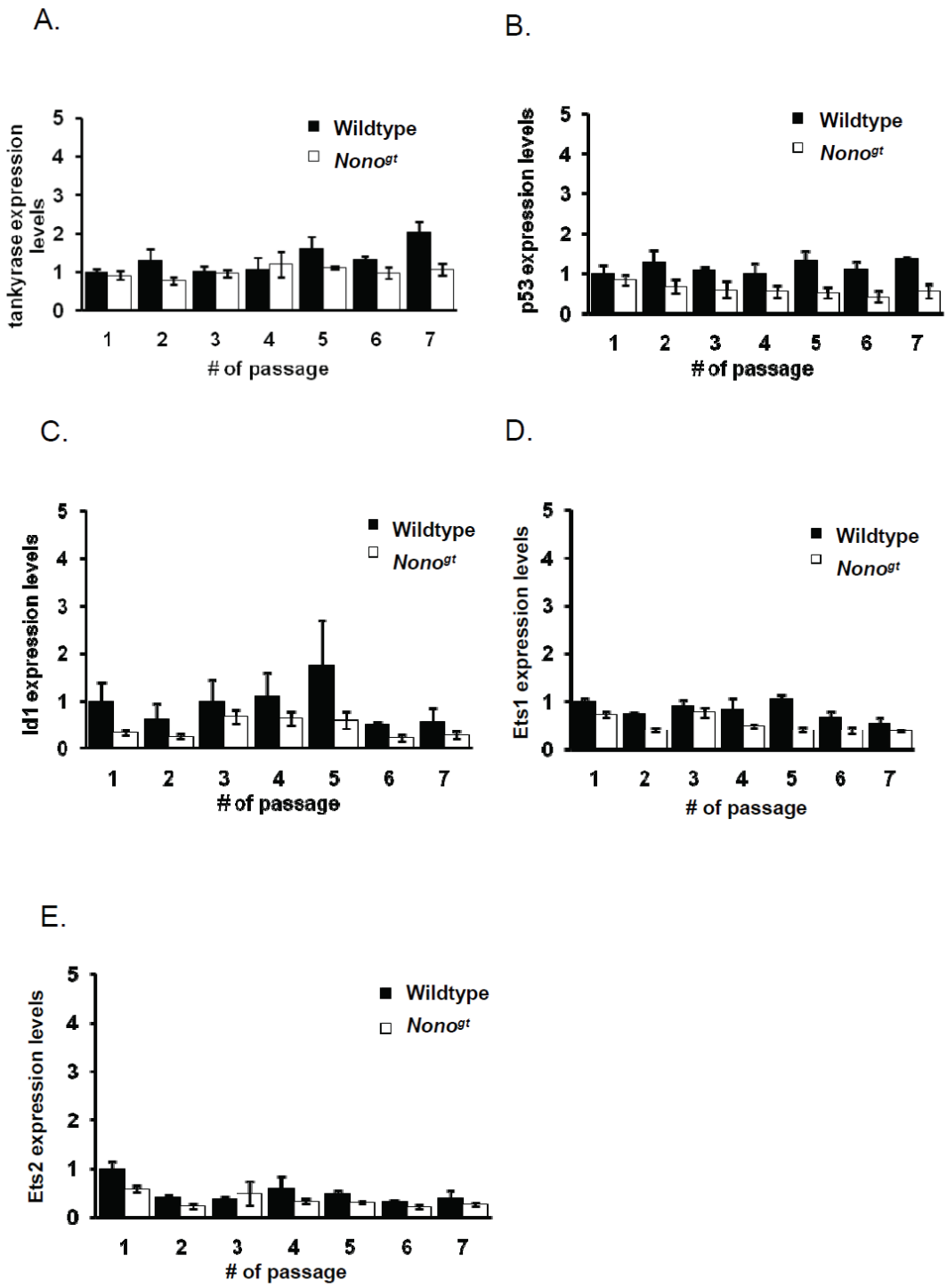

Fig. S1. Transcription of senescence-implicated genes in serially passaged wildtype and *Nono*^{gt} primary fibroblasts. WT and *Nono*^{gt} primary fibroblasts were counted and passaged every two days, and a constant number of cells plated to a new dish. Total RNA was harvested from cells in each passage, and qPCR was used to quantify the transcript levels of senescence-implicated genes *tankyrase* **(A)** and *p53* **(B)**, as well as upstream regulators of *p16-Ink4A*, the genes *Id1* **(C)**, *Ets1* **(D)**, and *Ets2* **(E)**. Values are plotted in arbitrary units relative to wildtype levels at passage 1.

Fig. S2

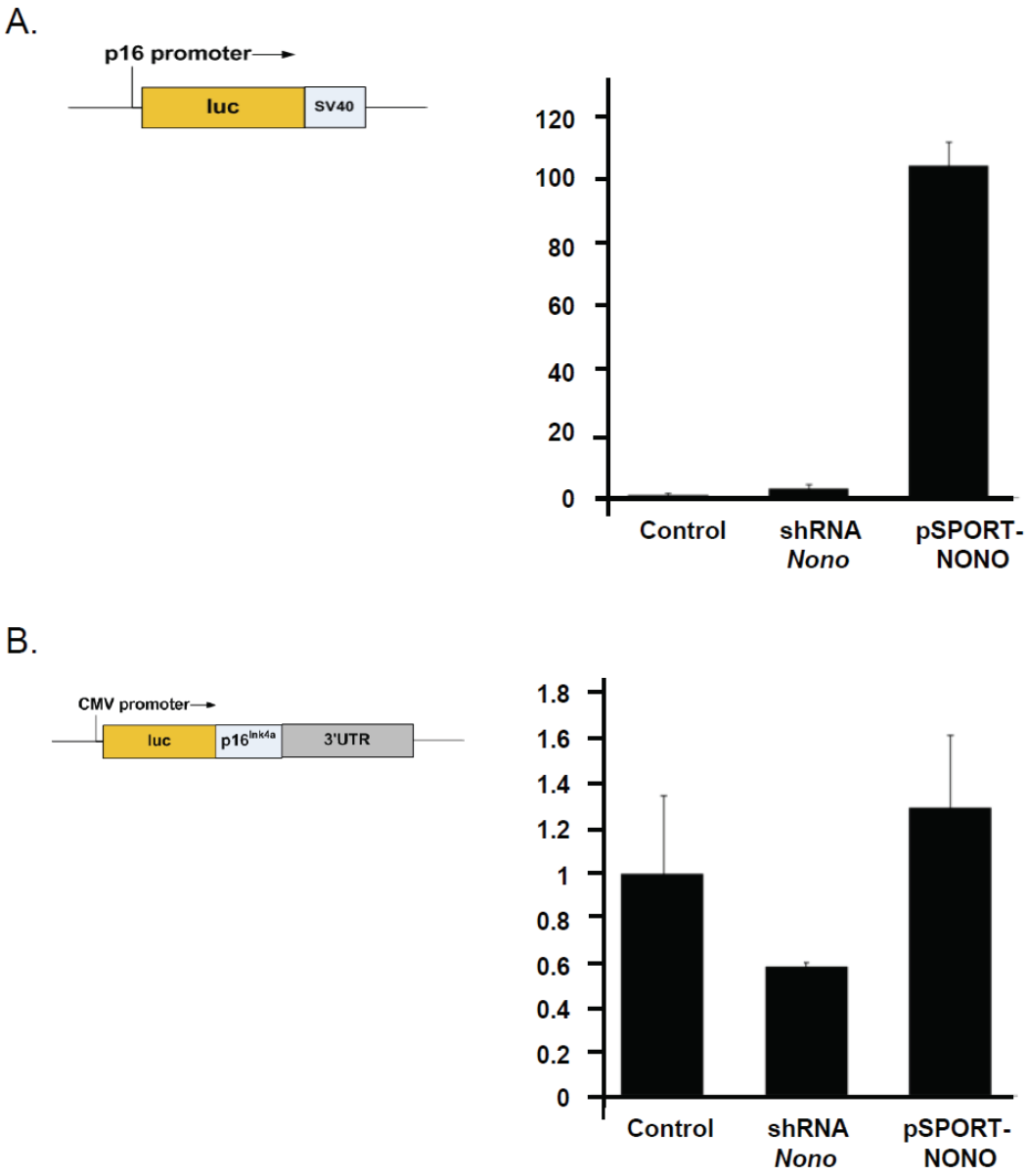

Fig. S2. NONO activates transcription of *p16-Ink4A* promoter reporters. (A) Relative expression levels of the diagrammed *p16-luc* promoter construct alone, in the presence of a NONO-targeting RNAi hairpin that reduces NONO levels 10x, and in the presence of a NONO-overexpressing vector. **(B)** Similar experiments using the diagrammed construct containing the *p16-Ink4A* 3'-UTR.

Fig. S3

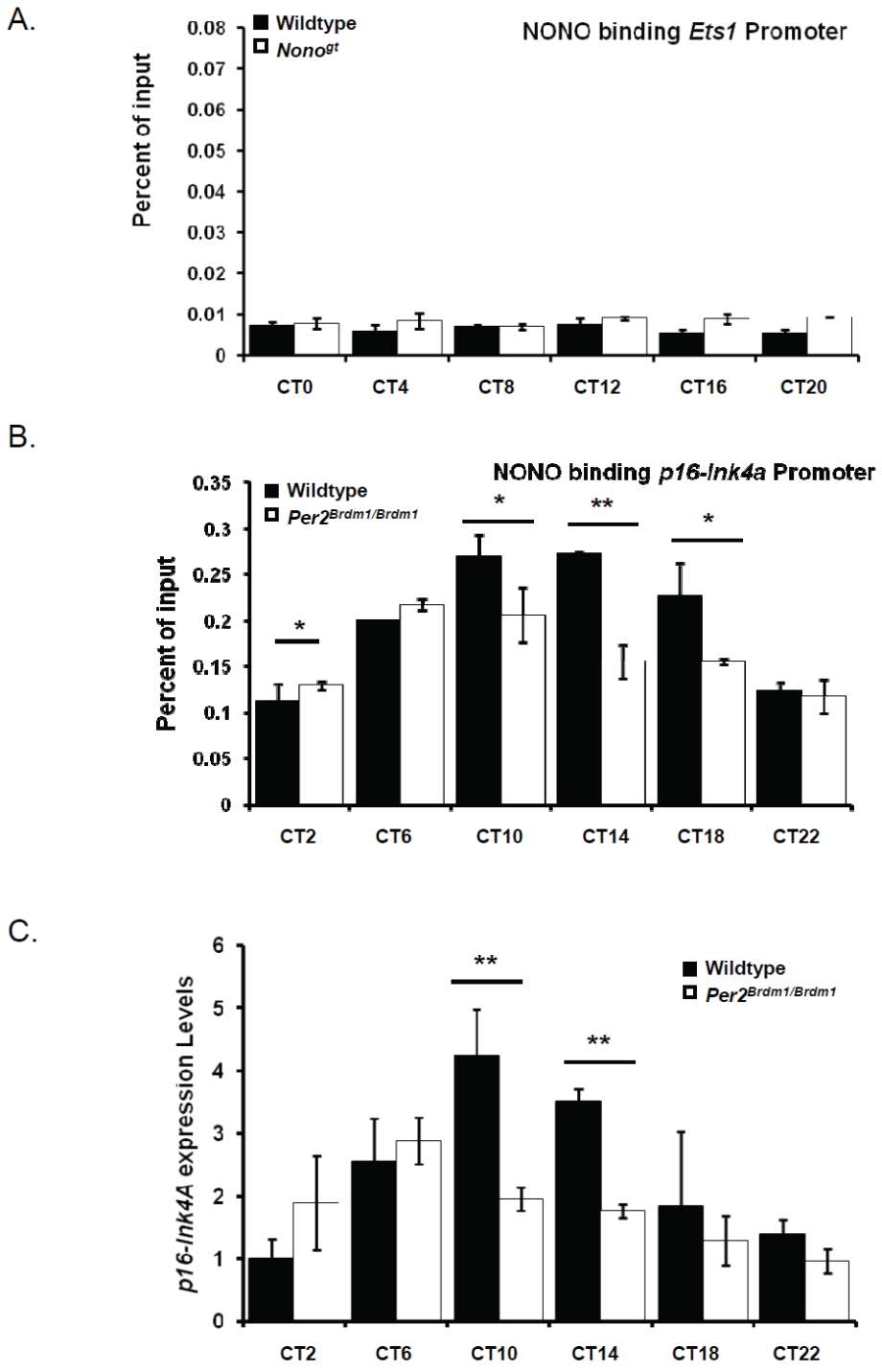

Fig. S3. **(A)** Liver chromatin was harvested from WT and *Nono*^{gt} mice at different times of day, and subject to immunoprecipitation using an anti-NONO antibody. Genomic DNA was purified from the precipitate, and DNA from the *Ets1* promoter was quantified by qPCR. **(B)** ChIP analyses similar to those in (A) were conducted, but using chromatin harvested from WT and *Per2*$^{brd1m/brdm1}$ mice, and then precipitated with an anti-NONO antibody. DNA from the *p16-Ink4A* promoter was quantified by qPCR. **(C)** RNA was harvested from the livers in (B), and *p16-Ink4A* transcript levels were quantified by qPCR.

Fig. S4

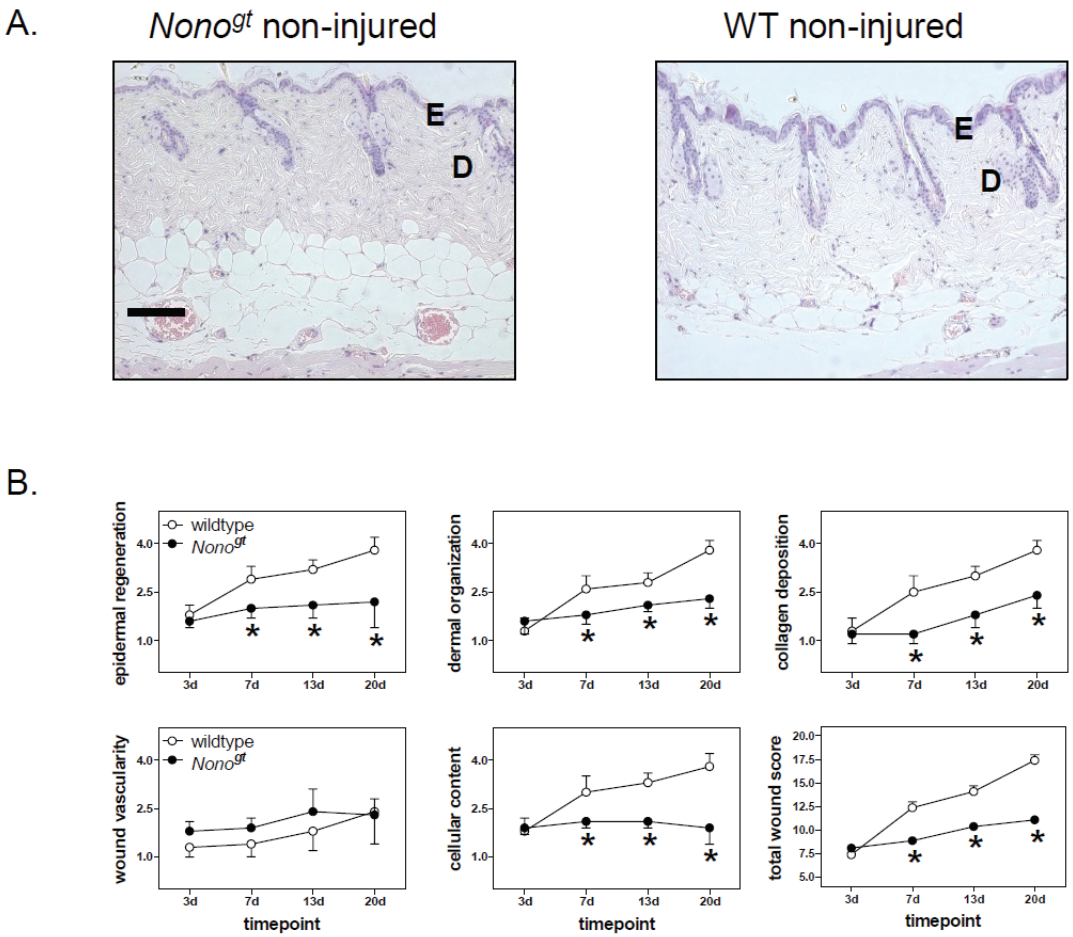

Fig. S4. Dermal structure and incisional wound healing in wildtype and *Nono*^{gt} mice. **(A)** H/E stained paraffin sections from un-injured dorsal skin. Normal skin morphogenesis is not affected in *Nono*^{gt} mice compared to wild type (WT) littermates (10-11 weeks of age, 24g body weight). **(B)** Full wound healing subscores of normal healing WT mice compared to *Nono*^{gt} mice on day 3, 7, 13 and 20 after incisional wounding in the dorsal skinfold. Data represent means ± SD (n=5).

Table S1

Gene	Fold Difference NONOgt/Wildtype
Abl1	0.69
Ak1	0.48
Apbb1	0.55
Atm	1.05
Brca1	0.96
Brca2	1.30
Camk2a	1.39
Camk2b	0.89
Casp3	0.86
Ccna1	1.15
Ccna2	1.52
Ccnb1	1.17
Ccnb2	0.82
Ccnc	0.55
Ccnd1	0.70
Ccne1	1.46
Ccnf	1.32
Cdc25a	1.17
Cdk2	0.64
Cdk4	1.02
Cdk5rap1	1.11
Cdkn1a	0.74
Cdkn1b	0.87
Cdkn2a	0.64
Chek1	1.00
Cks1b	1.20
Ddit3	1.50
Dnajc2	0.82
Dst	1.56
E2f1	0.76
E2f2	0.73
E2f3	0.49
E2f4	1.11
Gadd45a	0.57
Gpr132	2.48
Hus1	1.00
Inha	0.82
Itgb1	0.81
Macf1	1.00
Mad2l1	1.55
Mcm2	0.98
Mcm3	0.87
Mcm4	0.83
Mdm2	0.46
Mki67	1.50
Mre11a	0.81

Gene	Fold Difference NONOgt/Wildtype
Msh2	0.85
Mtbp	1.54
Myb	0.87
Nek2	1.89
Nfatc1	0.67
Notch2	0.48
Npm2	0.83
Pcna	0.86
Pes1	0.62
Pkd1	1.02
Pmp22	0.64
Ppm1d	0.80
Ppp2r3a	0.70
Ppp3ca	0.39
Prm1	1.24
Rad17	0.78
Rad21	1.30
Rad51	0.73
Rad9	1.18
Ran	0.84
Rbl1	0.93
Rbl2	0.30
Sesn2	1.01
Sfn	1.01
Shc1	0.68
Skp2	0.91
Slfn1	1.02
Smc1a	0.90
Stag1	0.78
Sumo1	0.71
Taf10	0.99
Terf1	0.88
Tfdp1	1.15
Psmg2	1.03
Trp53	1.11
Trp63	1.02
Tsg101	0.60
Wee1	0.57
Gusb	0.69
Hprt1	1.20
Hsp90ab1	0.88
Gapdh	1.15
Actb	1.17

Table S1. **Regulation of cell cycle genes in wildtype and *Nono*^{gt} fibroblasts.**
Total RNA was harvested from dividing cultures of WT and *Nono*^{gt} primary fibroblasts, and subjected to qPCR array analysis. All array targets are shown, with fold-regulation of *Nono*^{gt} *vs.* WT fibroblasts.

Material and Methods

Plasmids

The bioluminescence reporter construct *pBmal1-Luciferase* and the NONO-targeting siRNA6 have been described previously (*9*). For NONO overexpression, a commerciallyobtained pSPORT construct was used (Clone ID 360A935 from Open Biosystems). p16-promoter-luciferase plasmid (D-761) and minimal p16 promoter plasmid (D-141) (*33*) were obtained from B.A. Mock and S. Zhang (Laboratory of Genetics, Center for Cancer Research, NCI, NIH, Bethesda, USA).

Animal husbandry

Chimeric mice were obtained from *Nono*^{gt} ES cells (C57Bl6 genotype) via standard blastocyst injection into SV129 mice by the University of California, Davis. Individual chimeric mice were back-crossed 4-10 generations against C57Bl6. All experiments were performed with littermates. Animal housing and experimental procedures are in agreement with veterinary law of the canton of Zurich. Genotyping was done as described at http://www.mmrrc.org/strains/38/ctr_protocol.pdf using a NONO-specific primer set (sense 5'-TTA GGG GGC CGA ACT ACT TGA ATT G-3', antisense 5'-GGG CCG GGC AGA TTT ACT AGT TTT T-3'. qPCR primer sequences are listed in the quantitative real-time PCR section of the Material and Methods, below).

Primary cell isolation and culture

Primary adult dermal fibroblasts (ADFs) were taken from a 0.5cm piece of mouse tail that was cut into several small pieces by using a razor blade. Digestion occurred in 1.8ml DMEM containing 20% FBS, 1% penicillin/streptomycin and 1% amphotericin B supplemented with 0.7 units liberase blendzyme, at 37°C and 5% CO2 for eight hours. After centrifugation in 1x PBS the pellet was resuspended in DMEM containing 20% FBS, 100U/ml penicillin, 100ug/ml streptomycin and 2.5ug/ml amphotericin B and kept at 37°C and 5% CO2. The day after medium was exchanged and remaining tail pieces were removed. Another medium exchange was done three days later. After a week the medium was exchanged for medium without amphotericin B. ADFs were cultured at 37°C and 5% CO2 in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin.

cDNA production, quantitative real-time PCR, and PCR arrays

RNA was extracted as described in *Current Protocols in Molecular Biology* (Wiley). 500ng of total RNA was transcribed to cDNA with SuperScript II (Invitrogen) using oligo (dT) primers according to manufacturer's instructions. For quantitative real-time PCR 20ng of cDNA was used and single transcript levels of genes were detected by Taqman probes used with the Taqman PCR mix protocol (Roche) using the AB7900 thermocycler. Primers used for detection of specific genes are listed below:

Gene	Orientation	Sequence (5' - 3')
Ets1	sense	CGG CAT CAT AGC ACA GTT CAA G
Ets1	antisense	CCC ATG CAA ACG GCT TTT AT
Ets1	probe	FAM-AAC CGC TAC CCG AAA CAT GGA AGA CTC AG-TAMRA
Id1	Primer Set	1) Assay ID: Mm00775963_g1
Ets2	Primer Set	1) Assay ID: Mm00468972_m1
NONO	sense	TGC GCT TCG CCT GTC A
NONO	antisense	GCA GTT CGT TCG ACA GTA CTG
NONO	probe	FAM-AGT GCA CCC TTA CAG TCC GCA ACC TT-TAMRA
qPCR		
p16-Ink4A	sense	CCC AAC GCC CCG AAC T
p16-Ink4A	antisense	GTG AAC GTT GCC CAT CAT CA
p16-Ink4A	probe	FAM-TTT CGG TCG TAC CCC GAT TCA GG-TAMRA

ChIP		
p16-Ink4A	sense	TTT CGC CCA ACG CCC CGA A
p16-Ink4A	antisense	ACC CGA CTG CAG ATG GGA CAC
p16-Ink4A	probe	FAM- CGA ACT CTT TCG GTC GTA CCC CGA TTC-TAMRA
p53	sense	GCA TCC CGT CCC CAT CA
p53	antisense	GGA TTG TGT CTC AGC CCT GAA G
p53	probe	FAM-CAG CCT CCC CCT CTC CTT GCT GTC TTA-TAMRA
tankyrase	sense	CGG CAG CAG AGC AGA AGA C
tankyrase	antisense	TGT ACT CCA GTT GCA GGT TTG AAT
tankyrase	probe	TAG TGA CCA CCC CTG GTA AAG GCC AGA-TAMRA
GAPDH	sense	CAT GGC CTT CCG TGT TCC TA
GAPDH	antisense	CCT GCT TCA CCA CCT TCT TGA
GAPDH	probe	YAK-CCG CCT GGA GAA ACC TGC CAA GTA TG-TAMRA

1) TaqMan@ Gene Expression Assays from Applied Biosystems are tested, but sequences are not provided. Assay consists of primer forward, primer reverse and probe, as usual.

PCR arrays to quantify cell cycle components were performed according to the manufacturer's instructions (SA Biosciences, Array name: PAMM-020E) using 500ng of total RNA.

Western blotting and immunohistochemistry

Western blotting was performed using standard procedures (*Current Protocols in Molecular Biology*, Wiley). Equal loading and size detection using protein ladder was verified by Ponceau-S staining of membranes prior to probing. The probing of the primary anti-NONO antibody (affinity-purified polyclonal antibody raised by Charles River Labs using bacteriallyproduced NONO protein) was done at a 1:200 dilution. The probing of the secondary antibody was done at a 1:10'000 dilution for anti-rabbit IgG coupled to horse-radishperoxidase (Sigma) and 1:1000 for anti-mouse HRP (Sigma) respectively.

Cellular senescence and cytometric measurements

For growth curves, primary fibroblasts from WT and *Nono*^{gt} animals were split every other day, plating each time 1x106 cells so that confluence was never reached. Within these plates, a coverslip was laid for senescence-associated β-galactosidase staining, and nonplated cells were saved for RNA isolation. Population doublings were calculated as the logarithm of the number of cells counted at the current passage divided by the number of cells at the first passage, adjusted for plating ratios. Senescence-associated β- galactosidase staining was performed using a senesence β-galactosidase Staining Kit (Cell Signaling Technology) following the protocol of the manufacturer. To measure cell cycle progression at different circadian times, circadian clocks in identical plates of WT and *Nono*^{gt} fibroblasts were synchronized with 100 nM dexamethasone for 15 minutes as described (*17*), and harvested at different times of day. Fixation and flow cytometric measurements of DNA content via propidium iodide staining were performed according to *Current Protocols in Cell Biology* chapter 8.4.4. For CFSE staining cells were trypsinized, resuspended in complete cell medium and then washed with 1xPBS. The pellet of cells was then resuspended in 1xPBS containing 5μM CFSE and incubated for 10 minutes at 37°C. By adding ice cold complete cell medium the reaction was stopped and the cells were washed with 1xPBS. The pellet of cells was resuspended in complete cel medium.

Transient transfections

For *p16Ink4A* reporter transfection studies lipofectamine LTX with PLUS reagent (Invitrogen) was used according to the manufacturer's instructions, cultivating cells in 24-well plates and transfecting them with a total of 662ng DNA of which 50ng were the promoter luciferase reporter construct, 12ng were pCMV-SEAP (Placental Alkaline Phosphatase), the internal normalization control. Varying amounts of pCMV-NONO plasmid were “balanced” by the addition of pKS(+) to a total of 600ng. Cells were harvested after 60 hours by washing once with 1x PBS and extracting luciferase with a luciferase assay kit (Promega) and normalized against total protein amount quantified by coomassie blue staining.

Chromatin Immunoprecipitations
Chromatin from mouse liver and tissue culture cells was obtained as described previously (*34*). Equal amounts of precleared chromatin were incubated overnight at 4°C with 1 μl of anti-NONO antibody or anti-PER2 antibody. The capture of the DNA:protein complexes, the washing conditions and the purifiction of the DNA fragments prior to qPCR as well the control antibodies have been described (*35*). The region-specific primer/probe pairs are listed above.

Histological wound scoring
WT and *Nono*^{gt} mice were anesthetized intraperitoneally (i.p.) with a mixture of 90 mg/kg body weight (BW) ketamine hydrochloride (Ketavet®, Parke Davis; Freiburg, Germany) and 25 mg/kg BW xylazine hydrochloride (Rompun®, Bayer; Leverkusen, Germany). The dorsal region was shaved and treated with a depilatory agent (Pilca Perfect; Stafford-Miller Continental, Oevel, Belgium). Three full-thickness incisions (6 mm) perpendicularly to the dorsal midline were made at one anterior and two posterior dorsal sites, and the skin margins were closed with Steri-Strips (Steri-StripTM S Surgical Skin Closure, 3MTM; Minnesota, U.S.). Mice were sacrificed on day 3, 7, 13, and 20 after wounding, the wounds embedded according to standard procedures and stained with masson-goldner trichrome and hematoxylin eosin. A total of 16 animals (8 WT and 8 *Nono*^{gt}, n=2 for each timepoint) were wounded and killed on the corresponding timepoints and two additional WT and *Nono*^{gt} mice without any incisions served as controls. Subsequently, identical experiments were performed on 3 each of WT, *Bmal1*$^{-/-}$, and *Per1/Per2*^{mut} mice, with sacrifice on day after wounding. In all cases, wound scoring was based on the quality of dermal organization, epidermal regeneration, collagen deposition, cellular content, and wound vascularity. The criteria used as histological scores of wound healing are summarized below.

Score	dermal organization	epidermal regeneration	collagen deposition	cellular content	wound vascularity
1	25% thickness of granulation tissue compared to healthy tissue	no epithelial closure	gap without ingrowing collagen fibrils	low cell proliferation, mainly inflammatory cells	1-3 capillaries per visual field
2	50% thickness of granulation tissue compared to healthy tissue	strong hyperproliferative epithelium	gap with ingrowing collagen fibrils	predominantly inflammatory cells or dysfunctional fibroblasts, hyper-proliferation	4-6 capillaries per visual field
3	75% thickness of granulation tissue compared to healthy tissue	moderate hyperproliferative epithelium	no gap, but unstable adhesion	predominantly normal fibroblasts	7-9 capillaries per visual field
4	thickness of granulation tissue equal to healthy tissue	thickness and structure equal to normal epithelium	no gap, stable adhesion	low cell proliferation, mainly fibroblasts	>9 capillaries per visual field

Statistical Methods
Bar graphs: Student t-test for significant differences between control and experimental groups. In all figures, N=3-4 independent experiments, with each experiment conducted in technical duplicate. Data is plotted +/- SEM. Line graphs: For each experimental data set, linear regression was conducted to determine best-fit line describing the data from each independent experiment. Overall significance of differences in doubling (e.g. Fig 1B) or in accumulation rate (e.g. Fig 2B) were then determined using a student two-tailed t-test of slopes of the regression lines from each data set. In Fig 1B, data from two independent experiments is depicted, and data is plotted +/- SD. In Fig 2B, data from 4 independent experiments is depicted, and data is plotted +/- SEM.

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Author Contributions

E.K. characterized the *Nono*^{gt} mouse line and maintained the strain. P.B. did population counting, rescue experiment and β-galactosidase staining in primary fibroblast. P.B. and **E.K.** performed qRT-PCR experiments. J.R. conducted the ChIP experiments. **E.K.** performed cell doubling and cell stage FACS experiments in primary fibroblasts of different mutant mouse strains and **E.K.** and T.B. analyzed the data. P.B. and S.A.B. did transient transfection experiments in established and primary cell lines, respectively. **E.K.** performed the RT-PCR array. **E.K.** and D.H. performed wounding and wound harvest in different mouse strains. D.H. and C.C. did immunohistochemistry of wound slices and analyzed the data. A.M. did initially characterize the embryonic stem cell line that was used for blastocyste injection to get *Nono*^{gt} mice. T.B. contributed the Bmal1$^{-/-}$ mice for wound healing assay. U.A. contributed the *Per1/Per2*^{mut} mice for wound healing assay. **E.K.** designed figures. **E.K.** and S.A.B. wrote the paper. All authors made comments on the manuscript.

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Chapter 3.3

Members of the DBHS family interact with PERIOD proteins to mediate circadian transcriptional feedback (Paper 3)

This section is submitted as:

Members of the DBHS family interact with PERIOD proteins to mediate circadian transcriptional feedback.

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Abstract

Factors interacting with core circadian clock components are essential to achieve transcriptional feedback necessary for metazoan clocks. Here we show that members of the DBHS family of RNA-binding proteins play such a role. Although all three DBHS proteins are members of nuclear paraspeckles and can potently repress transcription when recruited to DNA, only two of them – NONO and SFPQ – can interact with PER proteins and bind in circadian fashion directly to clock gene promoters to modulate transcription. As a consequence, mice or cells deficient in SFPQ or NONO show abnormal or abrogated circadian clocks, but those lacking the third paraspeckle factor PSPC1 or cells lacking paraspeckles themselves have normal clock function.

Key words: Circadian Clock, DBHS, NOPS, NONO, SFPQ, PSPC1, Paraspeckle, Transcription, Coregulator

Introduction

The circadian oscillator governs diurnal timing for most aspects of mammalian physiology. Its mechanism is cell-autonomous, and consists of interlocked feedback loops of circadian transcription, translation, and protein modification. In one loop, the CLOCK/NPAS2 and BMAL1/ARNTL transcriptional activators drive expression of the Period (*Per1* and *Per2*) and Cryptochrome (*Cry1* and *Cry2*) gene families, whose products subsequently multimerize and repress their own transcription. In a second loop, the transcriptional repressor REV-ERBα, whose transcription is also driven by CLOCK and BMAL1, represses the expression of *Bmal1* itself (Dibner et al. 2010). Beyond these "dedicated" clock genes, a large number of other factors are necessary to the circadian clock or for its regulation of physiology – kinases and phosphatases, chromatin modifying factors, and other proteins (Masri and Sassone-Corsi 2010, Reischl and Kramer 2011). We have shown previously that the NONO protein in mammalian cells (or its ortholog NonA in flies) plays such a role by affecting Period-mediated transcriptional repression via unknown mechanisms (Brown et al. 2005).

NONO (also known as p54nrb in humans) has two RNA-binding domains and has been shown to regulate a variety of processes outside the circadian clock (Shav-Tal and Zipori 2002). These include transcriptional activation and repression (Mathur et al. 2001, Ishitani et al. 2003), pre-RNA processing (Kaneko et al. 2007), and RNA transport (Kanai et al. 2004). For example, it has been shown to regulate the transcriptional activation of the TORC family of growth and metabolic factors (Amelio et al. 2007). In an apparently

unrelated nuclear function, it also mediates the nuclear retention of edited RNAs in nuclear paraspeckles, RNA holding structures (Prasanth et al. 2005). These structures contain the related factors NONO, SFPQ, and PSPC1, as well as the scaffolding RNA *Neat1* (Bond and Fox 2009*)*. Herein, we show that two of these factors, independent of paraspeckles themselves, play important roles in the circadian clock by binding directly to clock gene promoters to enable Period proteins to repress transcription.

Results and Discussion

To better understand the function of NONO in the circadian clock and in mammalian physiology, we constructed a NONO-deficient mouse by using a virally mediated genetrap localized to the intron preceding the *Nono* translational start site (Fig S1A, B). In wildtype mice NONO is expressed in most tissues including the brain suprachiasmatic nuclei,and the resulting *Nono*^{gt} mouse showed no expression of *Nono* RNA or protein in all tissues including brain (Fig S1C and data not shown). As a consequence, these mice also showed a twenty-minute reduction in circadian behavioral period (Fig 1A). This reduction was highly significant, but it was nevertheless far less dramatic than the phenotype that we have published previously when we originally isolated NONO as a PER-interacting protein (Brown et al. 2005).

Therefore, we verified the relevance of NONO *in vivo* by looking for its presence at the promoters of clock genes. Since we showed previously that NONO interacted with PER proteins, we guessed that it ought to be found at PER-regulated clock genes. Chromatin immunoprecipitation experiments confirmed that this was indeed the case: NONO interacted with the promoter of the *Rev-Erb*α gene in circadian fashion with the same kinetics as the PER1 protein (Fig 1B, top). This interaction was considerably reduced but surprisingly not absent in *Nono*^{gt} mice, which completely lack NONO transcript and protein (Fig 1B, bottom). Equivalent results were seen for the *Dbp* promoter (Fig S2A), and no binding was observed at the promoter of the antiphasic *Bmal1* gene (Fig S2B). Based upon the residual binding observed at the *Rev-Erb*α and *Dbp* promoters, we considered the possibility that NONO might be redundant with a homologous factor with which our antibody might weakly cross-react. Conserved domain analysis (CDART, (Geer et al. 2002)) showed that the other two known paraspeckle proteins, PSPC1 and SFPQ, shared both high homology with NONO and a similar domain architecture (Fig S2C, D), and we speculated that all three proteins might have similar functions in the circadian oscillator.

To test this idea, we transfected vectors expressing each of the three proteins into cultured cells together with a luciferase reporter under control of the circadian *Rev-Erb*α

gene promoter. After synchronizing circadian clocks in these transfected cells with the dexamethasone (Balsalobre et al. 2000), we monitored reporter bioluminescence in real time. Overexpression of any of the three proteins in 3T3 fibroblasts abolished circadian rhythmicity (Fig 1C), indicating that the gene dosage of these three proteins is crucial to circadian clock function

We next undertook loss-of-function experiments based upon RNA interference (RNAi), in which U2OS cells containing an integrated *Bmal1-luciferase* reporter were infected with lentiviruses expressing shRNAs targeting *Pspc1* or *Sfpq.* RNAi hairpins against SFPQ dampened circadian oscillations dramatically (Fig 2A) similar to what was observed previously for NONO (Brown et al. 2005), but those against PSPC1 lengthened it slightly without affecting amplitude (Fig 2B). Measurement of *Sfpq* and *Pspc1* RNA levels in these cells showed that these hairpins reduced expression of *Sfpq* 7-fold, and *Pspc1* 2.5-fold (Fig S3A). Effects upon circadian rhythmicity were also seen in 3T3 cells transiently transfected with the circadian *Rev-Erbα* promoter reporter together with RNAi hairpins targeting *Pspc1* or *Sfpq* (Fig S3B). In this case, immunofluorescence experiments suggested that these hairpins reduced expression of SFPQ 2-fold, and PSPC1 10-fold (Fig S3C).

Since the three NONO-related proteins are also the three known members of nuclear paraspeckles, we speculated that the paraspeckle itself might serve a circadian role. This subnuclear domain requires the nuclear noncoding RNA *Neat1*, probably as a scaffold, and depletion of *Neat1* has been shown to eliminate paraspeckles themselves (Chen and Carmichael 2009, Clemson et al. 2009). By transiently transfecting shRNAs complementary to *Neat1* into U2OS cells, we were able to deplete paraspeckles, measured by counting the number of punctate PSPC1 foci (Fig 2C). However, cotransfection of the circadian *Bmal1-luc* reporter showed that the circadian clock retained normal period length in these paraspeckle-depleted cells (Fig 2D), making it unlikely that paraspeckles *per se* play a role in the circadian oscillator. Therefore, it is probable that nucleoplasmic, non-paraspeckle-associated pools of NONO, SFPQ, and PSPC1 proteins were responsible for the circadian effects that we have documented.

We have shown previously that NONO interacts directly with Period proteins (Brown et al. 2005). To see whether this property is true of NONO homologs, we tested interactions between PER proteins and SFPQ and PSPC1. In keeping with our expectations, SFPQ interacted with both PER1 and PER2 when epitope-tagged versions of these proteins were transfected into cells (Fig 3A). PSPC1 interacted with neither protein (Fig 3A), even

though it did interact strongly with SFPQ (Fig 3B). To verify these interactions *in vivo*, we immunoprecipitated PER2 protein from liver nuclear extracts, and were able to detect both NONO and SFPQ (Fig 3C). Furthermore, similarly to NONO, SFPQ could also be immunoprecipitated at the *Rev-Erb*α promoter in a circadian fashion in liver nuclear extracts, but PSPC1 could not (Fig 3D). Similar results were seen at the *Dbp* promoter (Fig S4A).

Based upon our chromatin immunoprecipitations and biochemistry, NONO and SFPQ are present at clock gene promoters with exactly the same temporal profile as PER proteins, and interact directly with them. Hence, we reasoned that these proteins probably act as corepressors. To test this hypothesis explicitly, we transfected primary mouse fibroblasts with an E-box-driven luciferase reporter, together with the transcriptional activators CLOCK and BMAL1, and either NONO, PSPC1, or SFPQ. Both NONO and SFPQ repressed CLOCK-BMAL-mediated transcription from the reporter, and PSPC1 demonstrated conflicting results, initially activating and then repressing at higher concentrations (Fig 4A). PER proteins were necessary for these effects: when equivalent transfections were performed using fibroblasts from *Per1*$^{brdm/brdm}$ *Per2*$^{brdm/brdm}$ mice that lack functional PER proteins and circadian clocks (Zheng et al. 2001), repression was no longer observed, but instead weak activation (Fig 4A).

Although the results above are consistent with a role in repression, in other reports NONO and SFPQ have been reported by different investigators as either transcriptional coactivtors or corepressors (Mathur et al. 2001; Ishitani et al. 2003). To confirm that these factors are repressors at circadian promoters, we designed fusions of NONO, PSPC1, and SFPQ with the GAL4 DNA-binding domain in order to be able to recruit them to DNA independently of PER proteins. When cotransfected into 3T3 cells together with a hybrid GAL4-E-box-luciferase reporter, all three proteins strongly repressed CLOCK-BMAL-mediated transcription (Fig 4B), though they had no effect when similarly recruited to the strongly-expressed CMV promoter (Fig 4C).

Finally, in order to verify the relevance of these factors to the circadian clock *in vivo*, we constructed mice containing genetrap-based deletions of *Pspc1* and *Sfpq*, to match the *Nono*^{gt} mouse described earlier in this paper. Homozygous *Pspc1*-genetrapped mice showed fivefold reduction in *Pspc1* transcript levels in multiple tissues (Fig S5A), and no detectable levels of PSPC1 protein in liver nuclear extracts (Fig S5C). Although the *Sfpq* genetrap was homozygous lethal, after normalization heterozygous mice showed up to twofold reduction in both RNA and protein (Fig S5B, D). When tested for circadian wheel-

running behavior, these *Sfpq*$^{gt/+}$ mice also showed a shortening of period similar to that of *Nono*^{gt} (Fig 5A, B), as well as altered entrainment in a minimal-light "skeleton" photoperiod (Fig S6A-C). *Pspc1*$^{gt/gt}$ mice showed no abnormalities (Fig 5A, B, Fig S6). Consistent with the proposed repressive role of these factors, at the gene expression level, the *Rev-Erb*α promoter showed modestly increased expression in liver extracts from all three knockouts at the time (CT8-12) that coincides with binding of NONO and PER2 (Fig 5C). Interestingly, this time coincides with the peak of *Rev-Erb*α expression levels and the beginning of their decline, but not with maximum repression. Hence, it is possible that these factors are associated with the establishment of repression but not its maintenance. Similar but smaller gene expression effects were seen upon *Per2* transcript levels, and the expression of other clock genes remained mostly unchanged (Fig S7).

Because of their homologies, shared functions, and abilities to interact with one another, the three factors NONO, PSPC1, and SFPQ have recently been classified by multiple authors as a family of proteins: the NOPS family (for NOno and PSpc1, (Staub et al. 2004) or DBHS family (for Drosophila Behavior, Human Splicing; (Bond and Fox 2009). Our data point to another important role of these proteins within the circadian oscillator. They are consistent with the idea that NONO and SFPQ play an important role in transcriptional repression at circadian clock genes, thanks to their ability to interact with PER proteins. It is very likely that there is a context-sensitive component to the activities of these factors: in other reports, NONO and SFPQ have been reported as either activators or repressors (Mathur et al. 2001, Ishitani et al. 2003), and we have reported antagonism of PER-mediated repression by NONO previously (Brown et al. 2005). The latter experiments were performed with immortalized 3T3 cells. Since NONO and SFPQ are also involved in the DNA damage response and in cell division (Proteau et al. 2005, Salton et al. 2010), it is possible that the activity of these factors changes in immortalized or transformed cells. Using 3T3 cells, we see with SFPQ exactly what we saw for NONO: dose-dependent *activation* (Fig S4B), rather than the repression that we reported above in primary cells. (Under the same conditions, PSPC1 showed weak dose-dependent repression, also the opposite of primary cells, and transfection of *Neat1* had no effects; Figs S4C, D). To verify the conclusion that SFPQ and NONO are repressors in the circadian system, we have presented data in this publication both *in vivo* from target circadian genes in NOPS protein-deficient mice, and *in vitro* using either native proteins or GAL4-mediated recruitment. Both sets of results are consistent with repression by these factors at circadian promoters.

Surprisingly, PSPC1 appears to play no role in this repression. Nevertheless, its circadian expression pattern suggests that it could play a role in circadian output even if our experiments disfavor a role in central clock function. Moreover, all three DBHS proteins can interact with the others (Bond and Fox 2009). Therefore, interplay between them can probably produce a variety of synthetic phenotypes – for example, effects of both activation and repression by NONO and SFPQ when transfected in large quantities, or dominant-negative abrogation of clock function when PSPC1 is overexpressed.

Our results also demonstrate that this highly homologous family of proteins likely exists in at least two nuclear pools. One of these pools is present in paraspeckles, and appears to play no role so far in the circadian clock, though it may be critical for nuclear retention of edited RNAs as reported by others (Zhang and Carmichael 2001, Prasanth et al. 2005, Chen and Carmichael 2009). A second pool of NONO and SFPQ is nucleoplasmic, and appears to interact directly with promoter regions to modulate transcription at a variety of target genes. Previous reports have shown that SFPQ can recruit the chromatin-modulating transcriptional repressor mSIN3a (Mathur et al. 2001), and that NONO can bind directly to the RNA polymerase C-terminal domain important for regulated transcription (Emili et al. 2002). Hence, multiple mechanisms are likely to play a role. In any case, through their interaction with PER proteins, we show that these factors play an important role directly in the circadian oscillator by binding to clock genes and repressing their transcription, thereby helping to establish the transcriptional feedback that is the hallmark of metazoan circadian clocks.

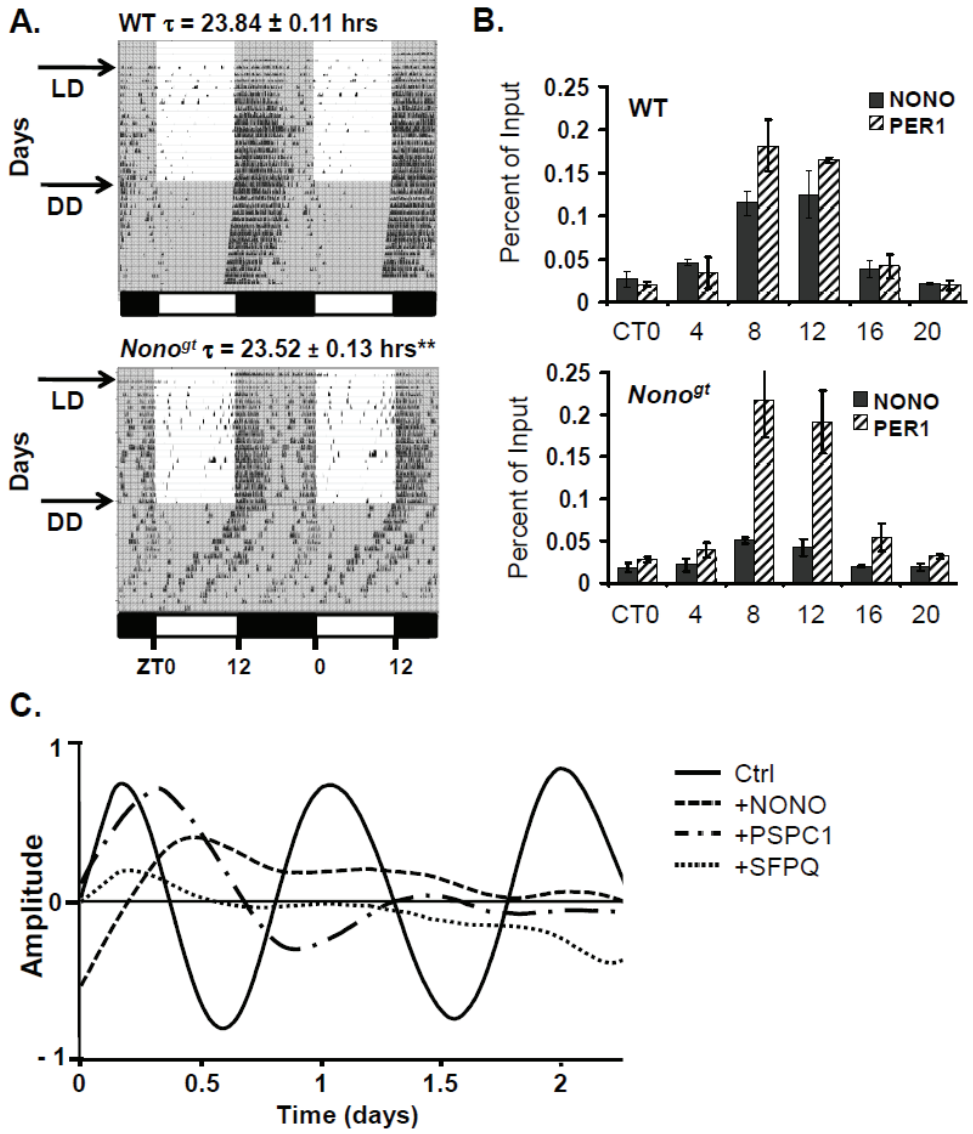

Fig. 1 **(A)** Wheel-running activity of wildtype and *Nono*^{gt} mice in 12:12 light-dark cycles (LD, arrow) and in constant darkness (DD, arrow). Darkness is indicated by grey shading. N=23. **(B)** Chromatin immunoprecipitation of NONO (black bars) and PER1 (striped bars) at the *Rev-Erb*α promoter in liver nuclei harvested at different times of day in constant darkness. CT0 = beginning of subjective day. Top panel, wildtype mice. Bottom panel, *Nono*^{gt} mice. (N=3 experiments, shown +/- standard deviation). **(C)** Bioluminescence from 3T3 cells transiently transfected with the *Rev-Erb*α*-luciferase* circadian reporter and constructs expressing either NONO, SFPQ, or PSPC1. Data shown is detrended and expressed in arbitrary units relative to mean expression. Solid black line, wildtype cells. Dashed line, cells overexpressing NONO. Dashed and dotted line, cells overexpressing PSPC1. Dotted line, cells overexpressing SFPQ.

Fig 2

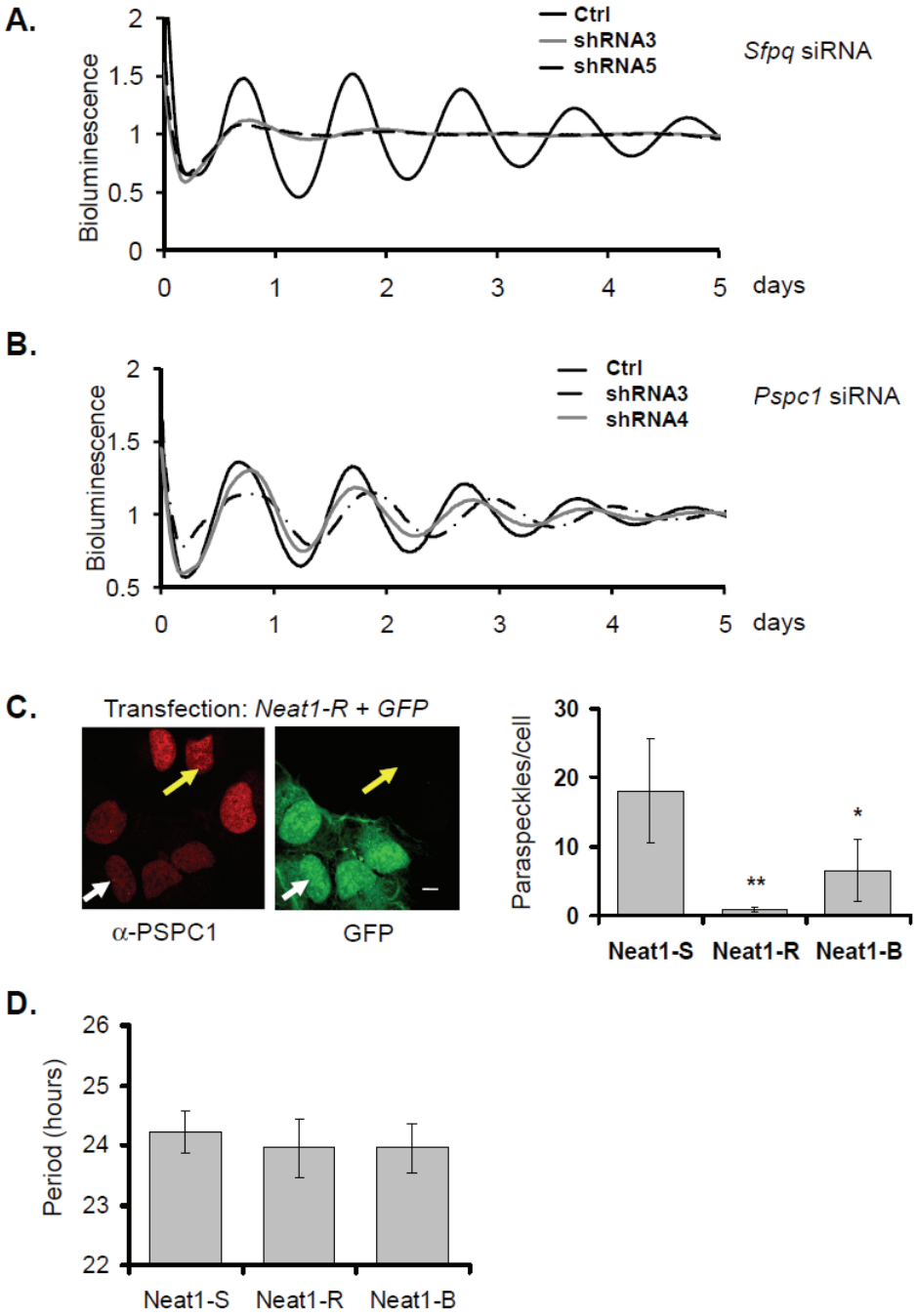

Fig. 2 (A) Bioluminescence from U2OS cells containing an integrated *Bmal1-luciferase* circadian reporter, infected with viruses expressing two different RNAi hairpins targeting the *Sfpq* gene, and then clock-synchronized with dexamethasone. Data shown is detrended and expressed in arbitrary units relative to mean expression. Solid black line, scrambled-sequence shRNA. Grey line, shRNA3. Dashed line, shRNA5. **(B)** Similar experiment with RNAi constructs targeting *Pspc1.* Dashed line, shRNA3. Grey line, shRNA4. **(C) Right,** Immunofluorescence from cells transfected with a plasmid expressing GFP and an RNAi interference construct targeting *Neat1 (Neat-R)*. Left panel, green filter (GFP). Right panel, same cells, red filter (PSPC1 protein). White arrow, paraspeckle in transfected cell; yellow arrow, paraspeckle in untransfected cell. Size bar, 10μm. **Left,** Quantification (+/- SD) of paraspeckles per cell for two different RNAi constructs (R and B), as well as a scrambled hairpin (S) stained similarly to (C), N= 12 cells (*Neat1-R)*, 24 (*Neat1- B)*, 18 (*Neat1-S).* Significance from Student t-test, *<0.05, **<0.01. **(D)** Period length of circadian reporter expression for U2OS cells cotransfected with the hairpins described in (C) and the *Bmal-luciferase* circadian reporter. (N=6 per sample, no significant differences as determined by Student t-test).

Fig 3

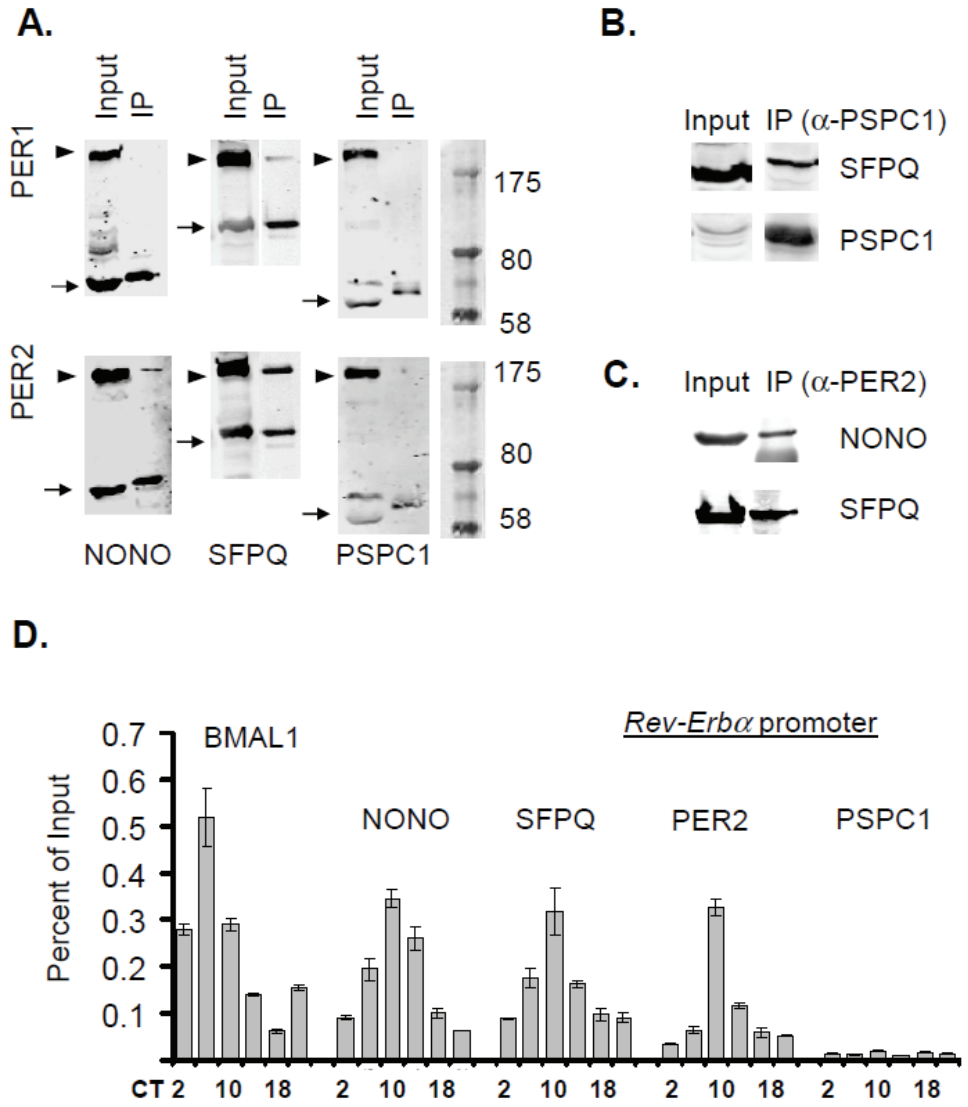

Fig. 3 **(A)** Immunoprecipitations from whole-cell extracts from 293T cells cotransfected with myc-tagged NONO, SFPQ, or PSPC1 (arrow) and Flag-tagged PER1 or PER2 (triangle). For each pair of lanes, left lane is 1/10 input, right is IP. All blots are probed with both anti-myc and anti-FLAG antibodies. **(B)** Mouse liver nuclear extracts from ZT16 were immunoprecipitated with anti-PSPC1, then probed with anti-PSPC1 or anti-SFPQ. Left lane 1/10 input, right lane IP. **(C)** Identical experiment to (B), but immunoprecipitated with anti-PER2 and probed with anti-NONO or anti-SFPQ. **(D)** Chromatin immunoprecipitation of the indicated proteins at the *Rev-Erbα* promoter in liver nuclei harvested at different times of day in constant darkness. N=4, +/- SE.

Fig 4

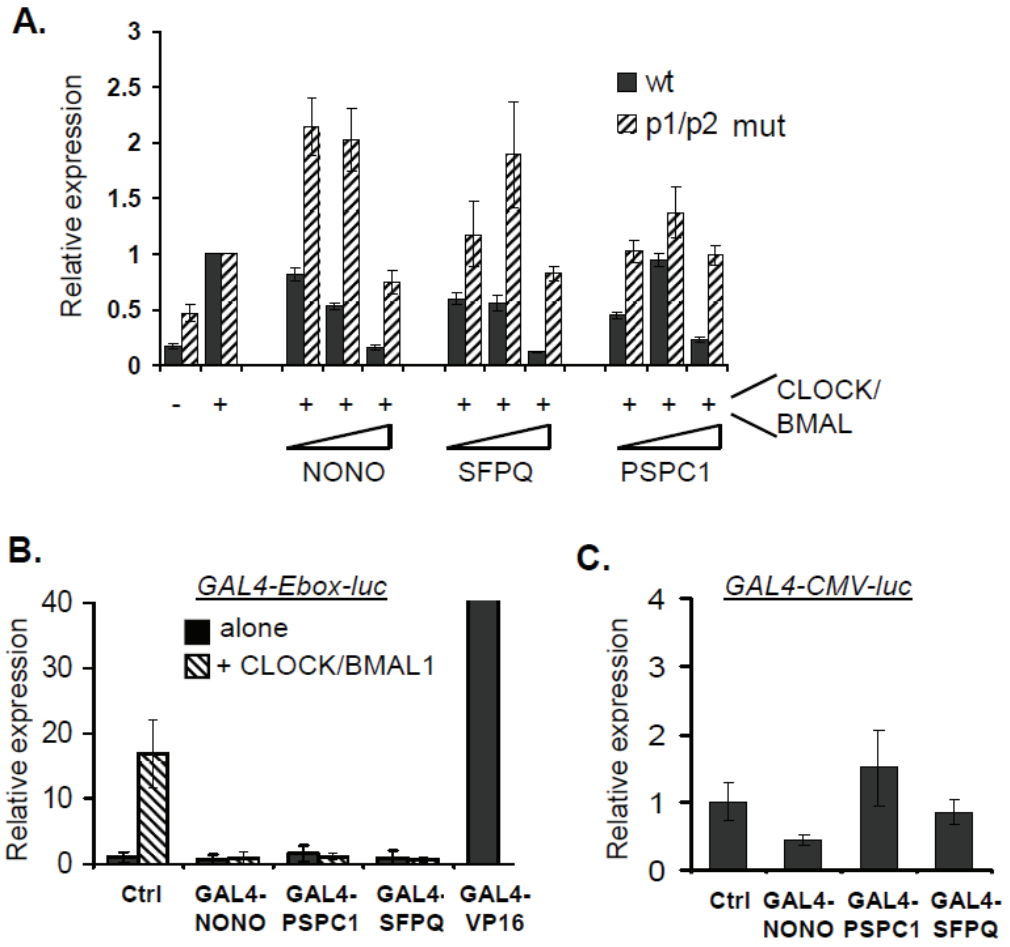

Fig. 4 **(A)** Bioluminescence measured after transient transfection of mouse primary fibroblasts from wildtype (black bars) or *per1*$^{brdm/brdm}$/*per2*$^{brdm/brdm}$ double mutant animals (striped bars) transfected with an *E-box-luciferase* reporter, and vectors expressing CLOCK and BMAL proteins, and NONO, SFPQ, or PSPC1 as indicated. N=3 experiments in duplicate, +/- SE, for all of figure **(B)** Transient transfection of 3T3 cells with a *GAL4-Ebox-luciferase* reporter and vectors expressing GAL4-NONO, -PSPC1, -SFPQ, or –VP16. Black bars, no exogenous activator added. Striped bars, vectors expressing CLOCK and BMAL1 also added. **(C)** Identical experiments using a *GAL4-CMV-luciferase* reporter and no exogenous activator.

Fig 5

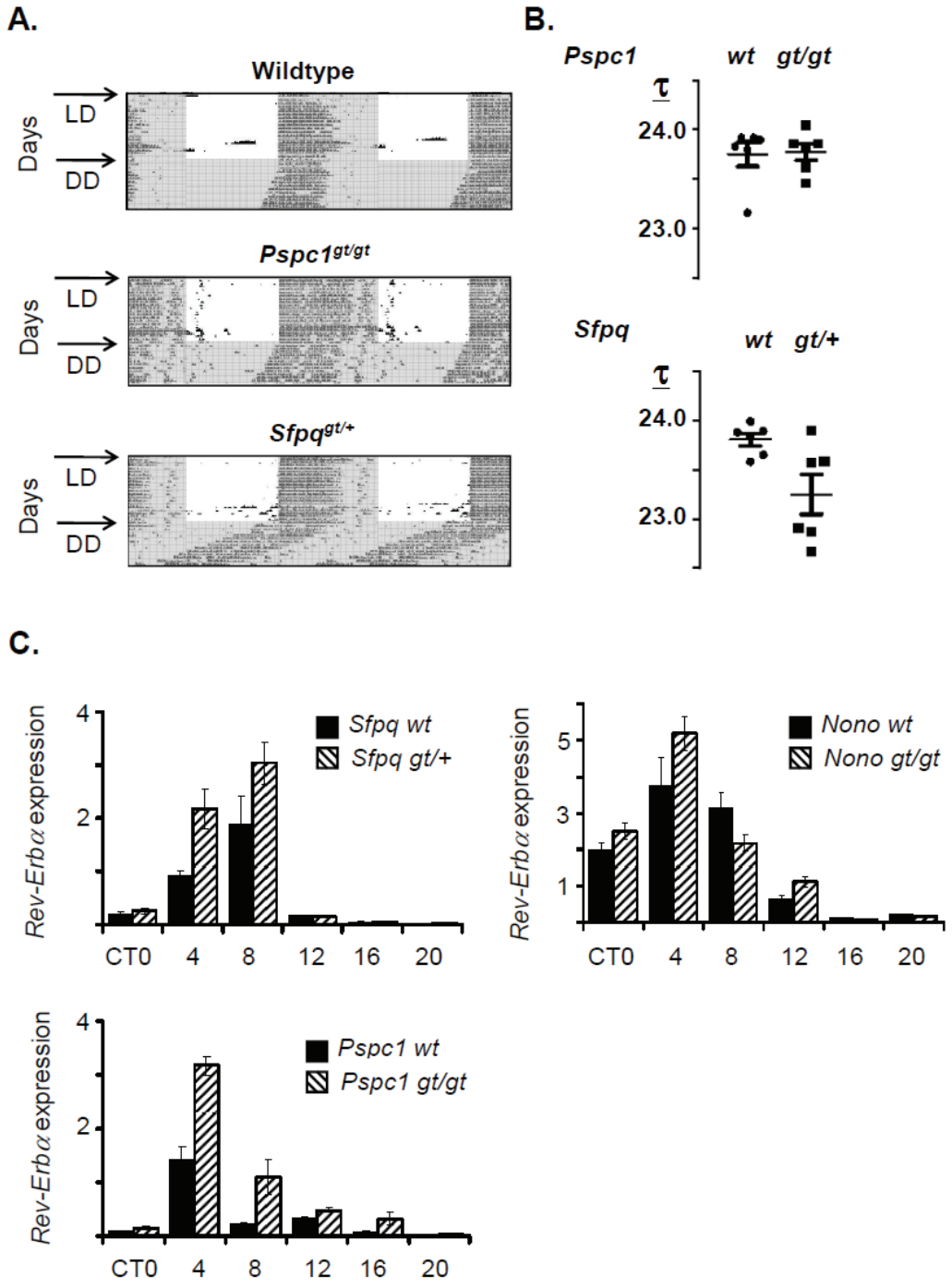

Fig. 5 **(A)** Left, wheel-running activity of wildtype, *Pspc1*$^{gt/gt}$, and Sfpq$^{gt/+}$ mice in 12:12 LD (arrow) and in constant darkness (DD). Darkness is indicated by grey shading. **(B)** Period lengths of six mice of each genotype, together with wildtype littermates. For *Pspc1,* p=0.36 (no significant difference); for *Sfpq*, p=0.01, using Student t-test. **(C)** *Rev-Erbα* RNA expression from *Nono, Sfpq,* and *Pspc1*-genetrapped mice (striped bars) and wildtype littermates (black bars), measured by qPCR from liver extracts harvested at different times of day from mice in constant darkness. N=2 mice per time point, RNA measured 4x in technical duplicate. Data shown is +/- SE.

Supplementary Figures

Fig. S1

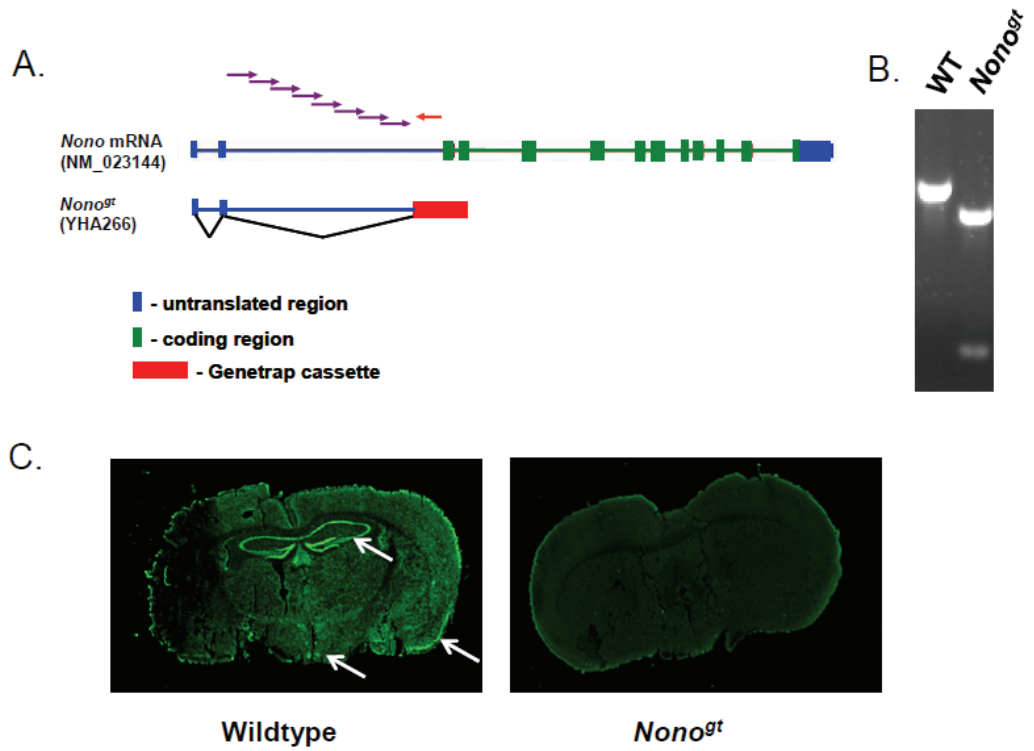

Fig. S1 **(A)** Scheme of *Nono* genetrap embryonic stem cell clone YHA266. Purple arrows correspond to primers designed along 2nd Intron to map the β-galactosidase insertion reporter construct (red arrow). Below are diagrammed the transcript formed, and its splicing pattern. Red primers were used in part B. **(B)** PCR from DNA harvested from wildtype and *Nono*^{gt} mouse fibroblasts, demonstrating the truncated gene product in mutants. **(C)** NONO protein expression in brain coronal sections from wildtype and *Nono*^{gt} animals, visualized by immunohistochemistry using a polyclonal anti-NONO antibody. Arrows from left to right show principal areas of NONO expression in wildtype mouse brain: suprachiasmatic nuclei, hippocampus, and neocortex.

Fig S2

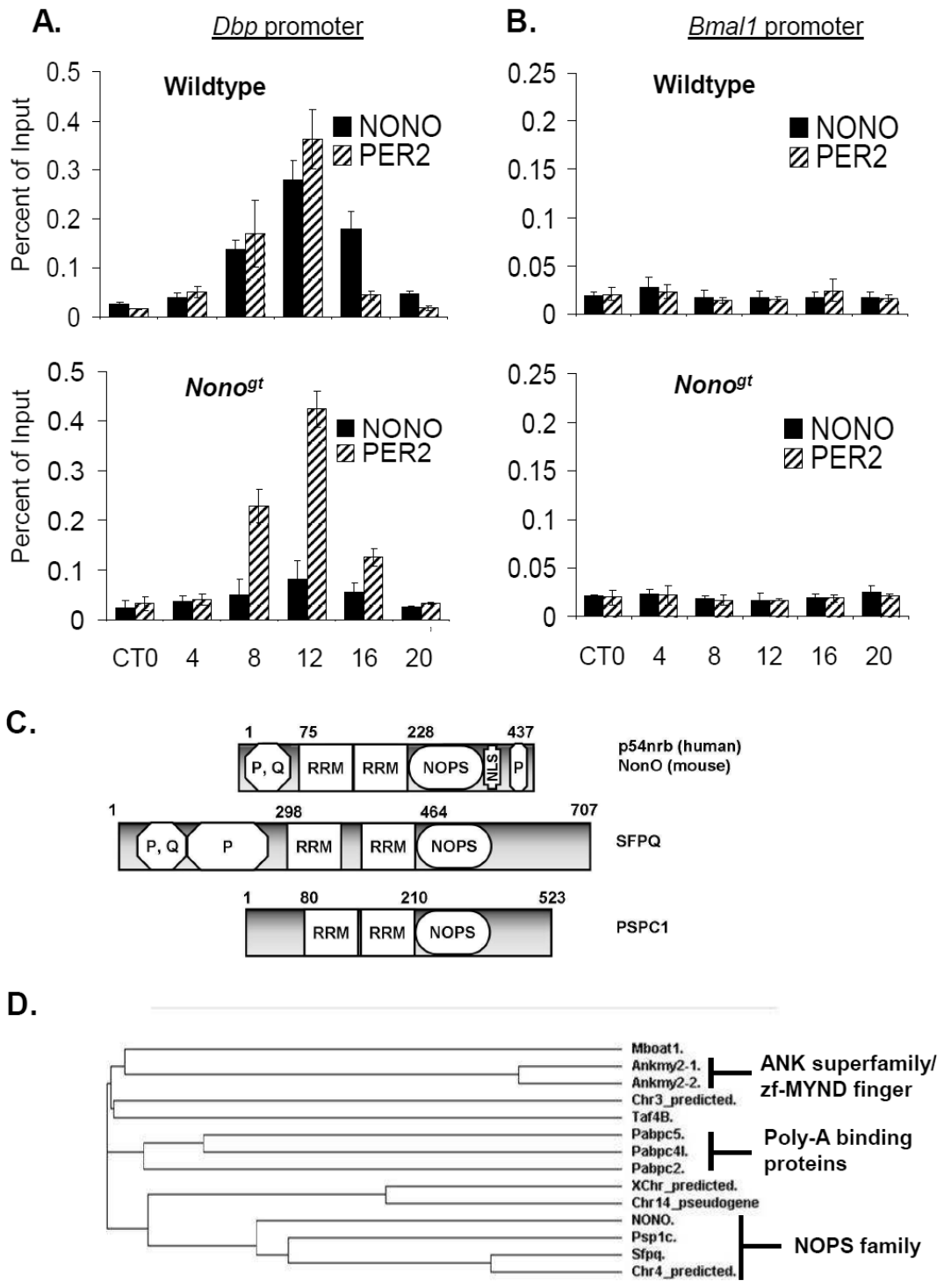

Fig. S2 **(A)** Chromatin immunoprecipitation of NONO (black bars) and PER2 (striped bars) at the *Dbp* promoter in liver nuclei harvested from wildtype mice (top panel) and *Nono*^{gt} mice (bottom panel) at different times of day in constant darkness. N=3 experiments, +/-SD. **(B)** Identical experiments for the *Bmal1* promoter. **(C)** Domain structure of NONO, SFPQ, and PSPC1 proteins. P, Q = proline- and glutamine-rich regions; RRM = RNA recognition motifs; NOPS = NOPS domain; NLS = nuclear localization sequence. **(D)** Cladogram showing the relationship of the NONO family of proteins to other RNA-binding proteins.

Fig S3

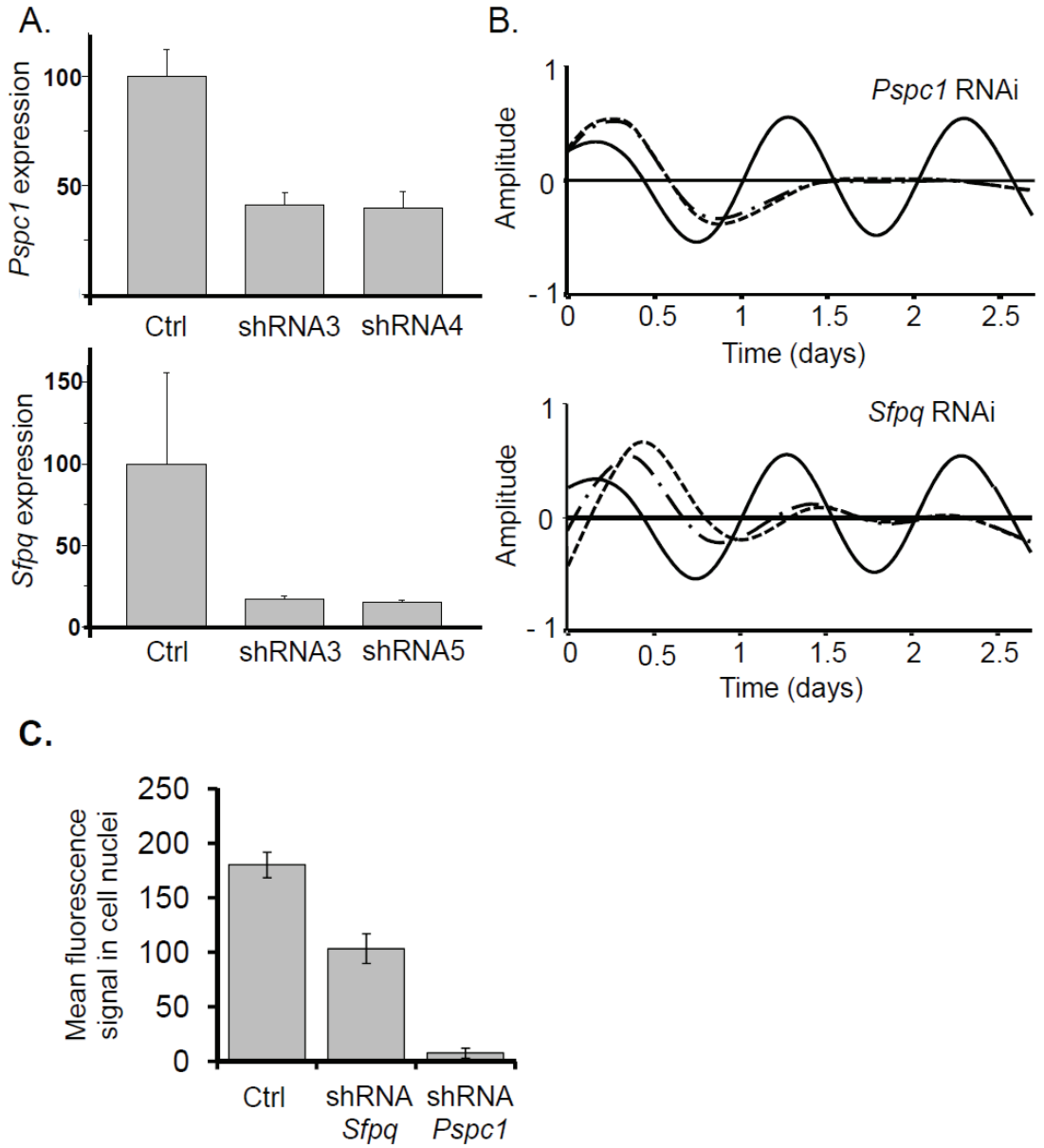

Fig. S3 (A) Transcript levels of *Pspc1* (top panel) and *Sfpq* (bottom panel) in U2OS cells infected with lentiviruses expressing the indicated RNAi targeting vectors used in Fig 2. N=3 +/- SE. (The same cells were used for this figure and for Figure 2.) **(B)** Bioluminescence from 3T3 cells transiently transfected with the *Rev-Erbα-luciferase* circadian reporter and RNAi constructs targeting either *Pspc1* (top) or *Sfpq* (bottom). After synchronization with dexamethasone, cultures were measured 3 days. Data are shown detrended and expressed in arbitrary units relative to mean expression. Solid black line, wildtype cells. Dashed lines, duplicate plates of cells expressing an *Sfpq*- or *Pspc1*-targeting vector. **(C)** Quantification of depletion of SFPQ and PSPC1 protein from experiments of Fig S3B. Relative repression from 3T3 cells cotransfected with a GFP-expressing plasmid and a plasmid expressing an RNAi interference construct targeting *Sfpq* or *Pspc1*. Averages shown are from 10 cells each, +/- SE. Mean fluorescence is expressed in arbitrary units.

Fig S4

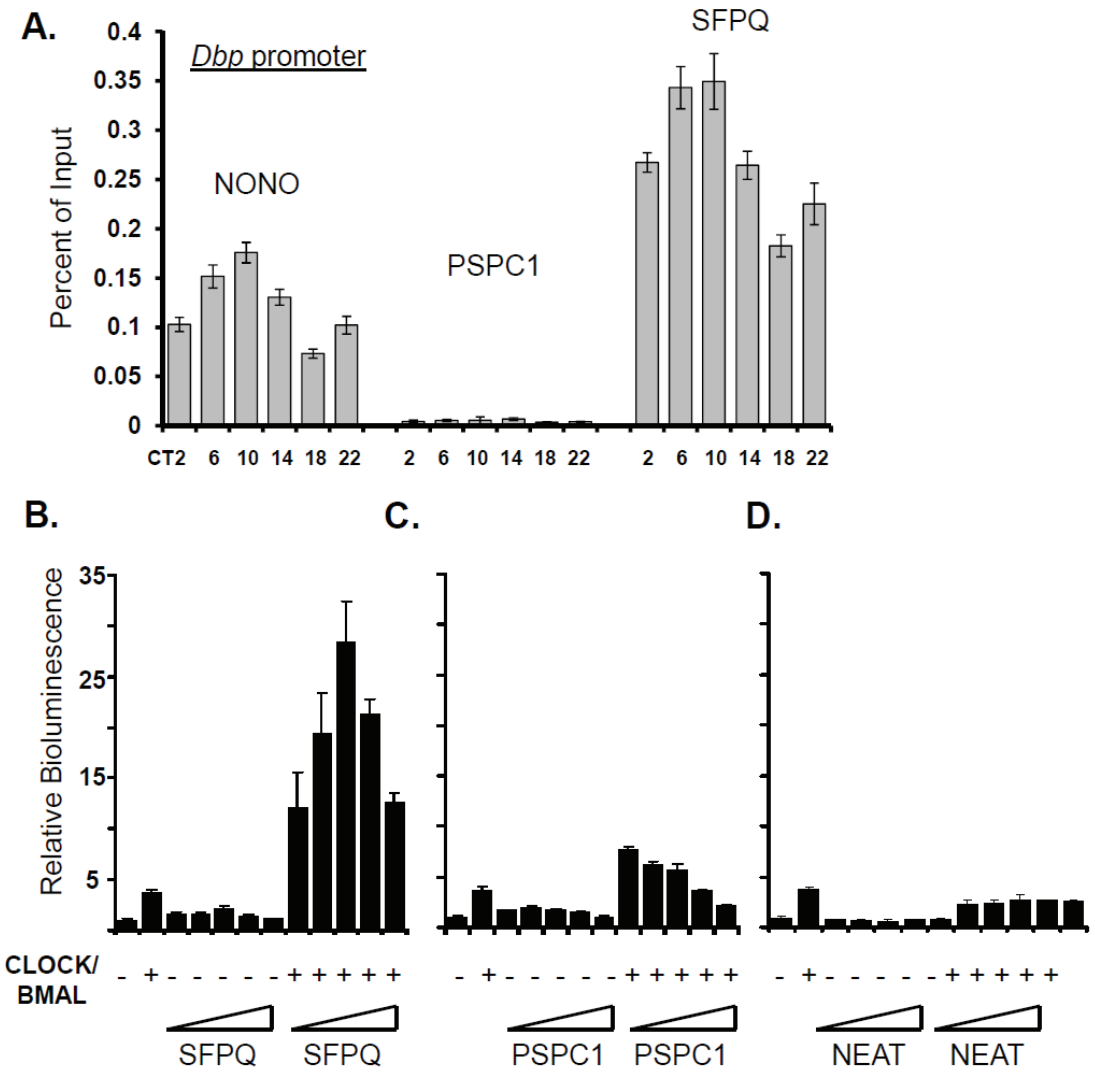

Fig. S4 **(A)** Chromatin immunoprecipitation of the indicated proteins at the *Dbp* promoter in liver nuclei harvested at different times of day in constant darkness. N=3 livers per timepoint, pooled. **(B)** Bioluminescence measured after transient transfection of 3T3 cells transfected with an *E-box-luciferase* reporter, and vectors expressing CLOCK and BMAL proteins and increasing amounts of SFPQ as indicated. N=6 experiments for parts B-D, +/- SE. **(C)** Similar experiments with a vector expressing PSPC1. **(D)** Similar experiments with a vector expressing *Neat1*.

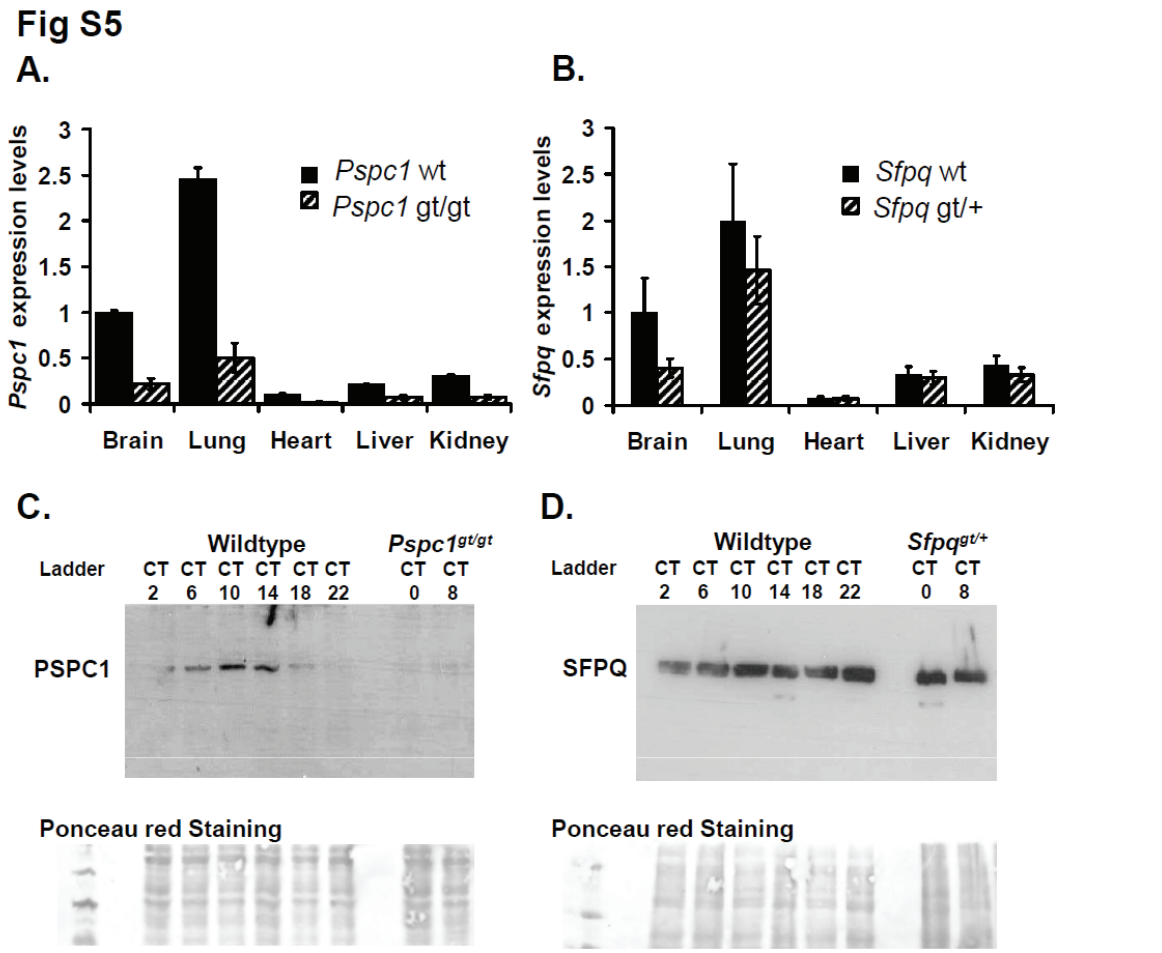

Fig. S5 **(A)** *Pspc1* RNA levels measured by qPCR from different tissues of genetrapped mice (striped bars) and wildtype littermates (black bars). For parts A-B, N=2 mice per measurement, measured 4x in duplicate, +/- SE. **(B)** *Sfpq* RNA levels measured by qPCR from different tissues of genetrapped mice (striped bars) and wildtype littermates (black bars). **(C)** PSPC1 protein levels in liver nuclear extracts harvested at different times of day from wildtype and genetrapped animals kept in darkness. Top panel, western blot probed with anti-PSPC1. Bottom panel, Ponceau-S staining of filter to show equal loading. **(D)** SFPQ protein levels in liver nuclear extracts harvested at different times of day from wildtype and genetrapped animals kept in darkness. Top panel, western blot probed with anti-SFPQ. Bottom panel, Ponceau-S staining of filter to show relative loading.

Fig S6

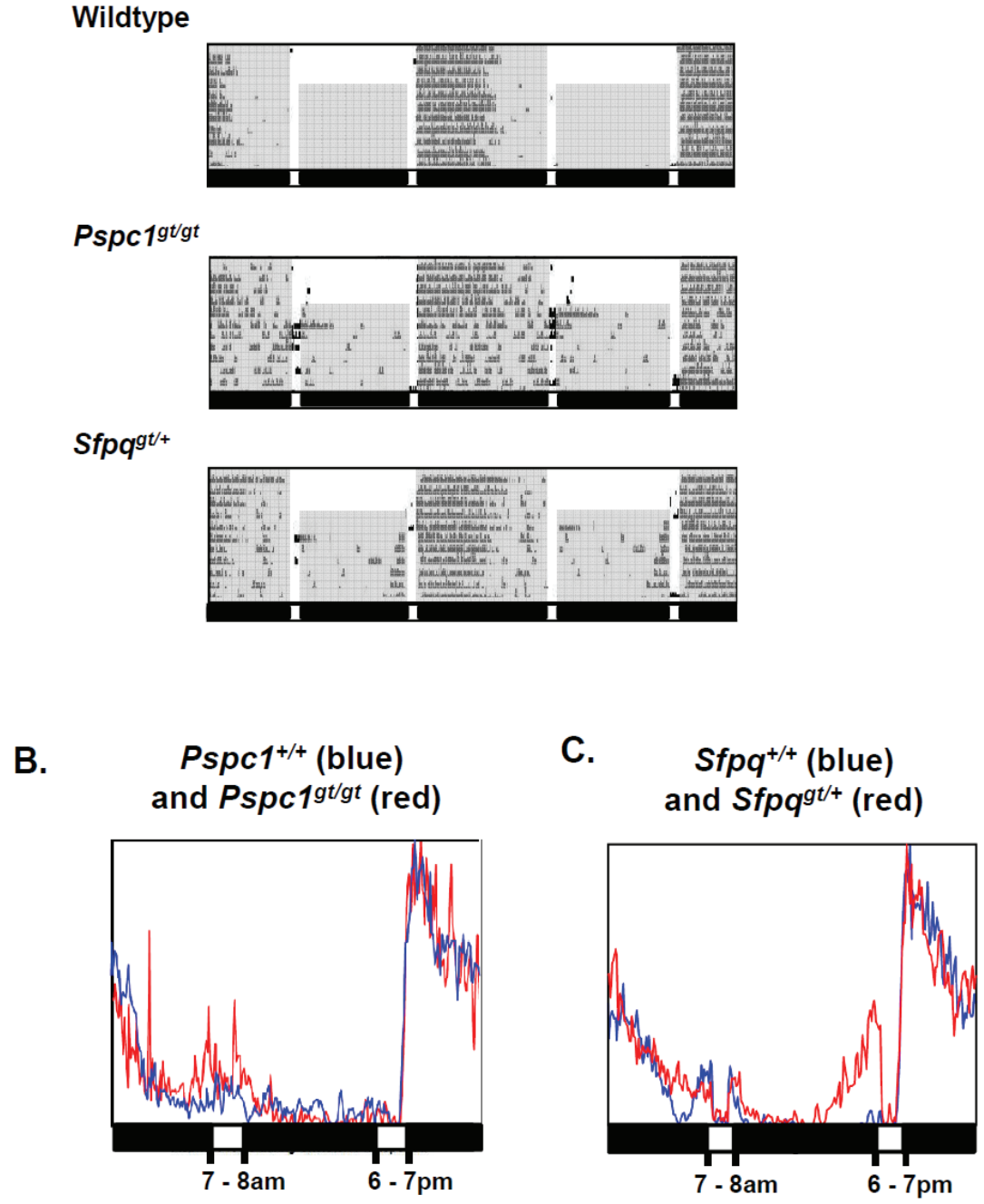

Fig. S6 **(A)** Wheel running activity during a skeleton photoperiod regimen of a representative wildtype, *Pspc1*$^{gt/gt}$, and *Sfpq*$^{gt/+}$ mouse. Grey shading, darkness. After a standard period of LD entrainment, mice received an hour-long light pulse at the normal time of lights-on (7-8am) or lights-off (6-7pm). **(B)** Averaged activity from 6 wildtype and 6 *Pspc1*$^{gt/gt}$ mice housed in a 1-hour-light skeleton photoperiod schedule. **(C)** Identical experiments for *Sfpq*$^{gt/+}$ animals.

Fig S7

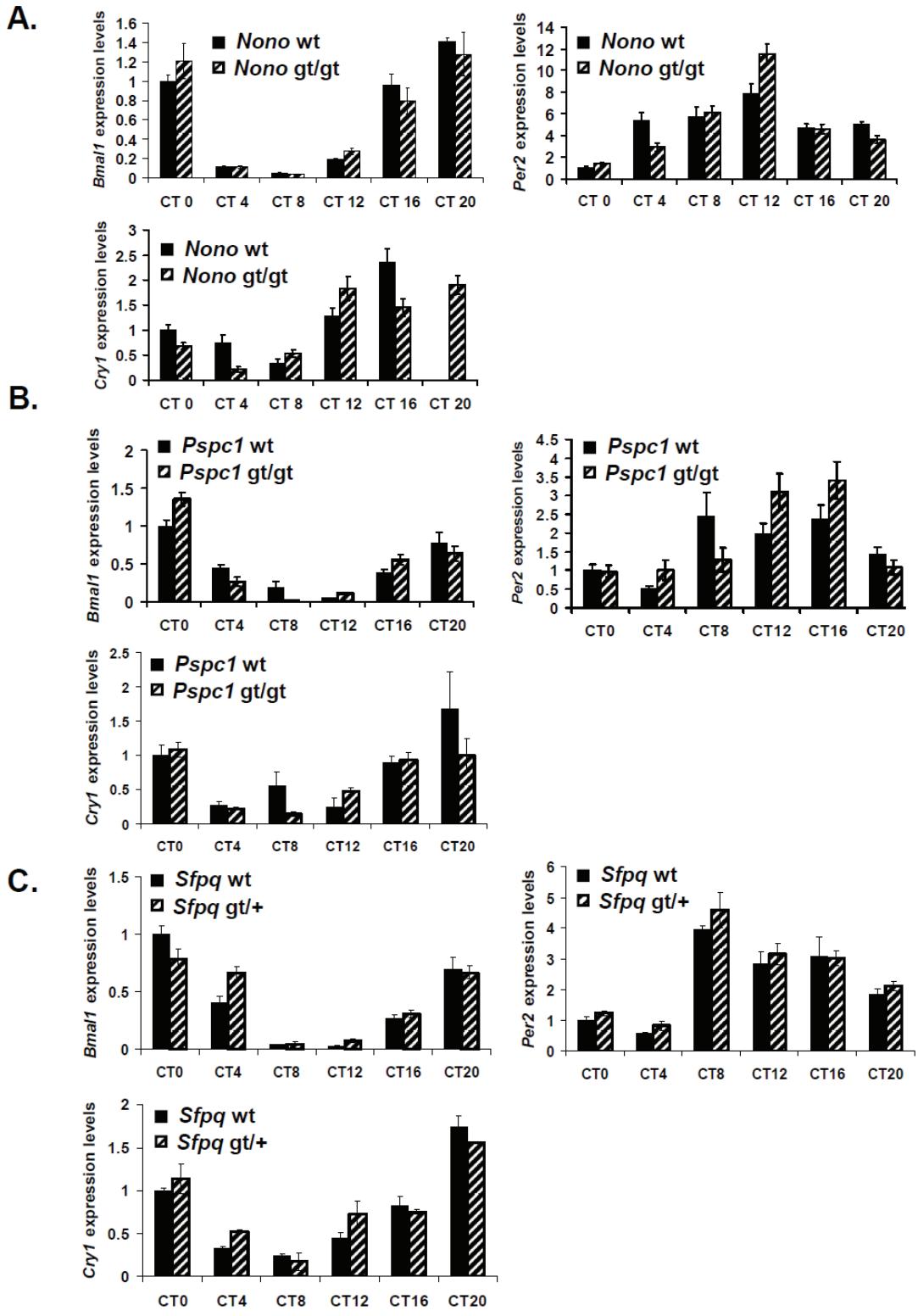

Fig. S7 RNA expression from *Nono, Sfpq,* and *Pspc1*-genetrapped mice (striped bars) and wildtype littermates (black bars), measured by qPCR from liver extracts harvested at different times of day from mice in constant darkness. Detected RNAs are indicated in each panel. N=2 mice per timepoint, measured 4x in duplicate, +/- SE. **(A)** *Nono*^{gt} mice and littermates. **(B)** *Pspc1*$^{gt/gt}$ mice and littermates. **(C)** *Sfpq*$^{gt/+}$ mice and littermates.

Material and Methods

Plasmids

The bioluminescence reporter construct *pBmal1-Luciferase* has been described previously (Nagoshi et al. 2004). Overexpression of NONO, SFPQ, and PSPC1 (tagged with the myc epitope) were achieved using the plasmids described in (Kuwahara et al. 2006). Plasmids expressing PER1 and PER2 proteins tagged with the FLAG epitope were a gift of T. Wallach (Kramer lab, Charite Universitätsmedizin, Berlin). To create GAL4 fusion constructs, the same constructs were obtained as entry (^{TM} Invitrogen) vectors from NITE (the Japanese Bioresource Information Center), and recombined into a destination (^{TM} Invitrogen) vector containing the GAL4 DNA-binding domain (aa 1-93). This vector was made by cloning PCRed recombination sites from pEF-DEST51 (Invitrogen) into pSCT-GALVP80 (gift of W. Schaffner, University of Zurich). The *Neat1* overexpression vector is described in Clemson et al. 2009. RNAi vectors against NONO have been described previously in Brown et al. 2005. Vectors targeting SFPQ and PSPC1 were purchased from Open Biosystems (clone numbers RRM3981 – 98064499 TRCN0000102241 and RMM3981 – 98064691 TRCN0000102470, respectively). p4xEbox-*luc* is described in Brown et al. 2005. pGAL4-Ebox-luc was made by inserting a multimerized 5xGAL4 site (cut from pFR-luc, Invitrogen) upstream of the E boxes in p4xEbox-luc. pGAL4-CMV-luc was made by inserting the same fragment the same distance upstream relative to the transcription start site of the CMV promoter.

Animal husbandry

Chimeric mice were obtained from *Nono*^{gt} ES cells (C57Bl6 genotype) via standard blastocyst injection of ES clone YHA266 into SV129 mice by the University of California, Davis. Individual chimeric mice were back-crossed 4-10 generations against C57Bl6. The same procedure was chosen to obtain *Pspc1*$^{gt/gt}$ and *Sfpq*$^{gt/+}$ mutant mice, using ES clones RRS358 and BC0256, respectively. Individual chimeric mice were back-crossed 2 generations against C57Bl6. All experiments were performed by comparing wildtype and mutant littermates. Animal housing and experimental procedures are in agreement with veterinary law of the canton of Zurich.

Animal activity measurements

For period measurements of *Nono*^{gt} mice, 24 mice of each genotype were habituated to a controlled 12:12 light-dark (LD) cycle in the presence of running wheels for 2 weeks, and then kept in constant dim red light for an additional two weeks. Data recording and period analysis was performed using the Clocklab software package (Actimetrics). Period measurements of *Pspc1*$^{gt/gt}$ and *Sfpq*$^{gt/+}$ mice were performed identically except that 6 mice of each genotype were used, and measurements were performed twice on each mouse. For skeleton photoperiod measurements, the same mice were given 1 hour of normal room light at each LD transition of a normal day, and otherwise kept in constant dim red light. Running wheel activity was measured as in period experiments, but plotted as the sum of activities of all the mice over a 24-hour day using the Clocklab software.

Primary cell isolation and culture

Primary adult dermal fibroblasts (ADFs) were taken from a 0.5cm piece of mouse tail that was cut into several small pieces by using a razor blade. Digestion occurred in 1.8ml DMEM containing 20% FBS, 1% penicillin/streptomycin and 1% amphotericin B supplemented with 0.7 units liberase blendzyme (Roche), at 37°C and 5% CO2 for eight hours. After centrifugation in 1x PBS the pellet was resuspended in DMEM containing 20% FBS, 100U/ml penicillin, 100ug/ml streptomycin and 2.5ug/ml amphotericin B and kept at 37°C and 5% CO2. The day after, medium was exchanged and remaining tail pieces were removed. Another medium exchange was done three days later. After a week the medium was exchanged for medium without amphotericin B. ADFs were cultured at 37°C and 5% CO2 in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin.

Transient transfections

For *p4xE-box* luciferase reporter transfection studies in NIH3T3 cells, lipofectamine LTX with PLUS reagent (Invitrogen) was used according to the manufacturer's instructions, cultivating cells in 24-well plates and transfecting them with a total of 850ng DNA of which 50ng were the promoter luciferase reporter construct. Varying amounts of plasmid were “balanced” by the addition of pcDNA3.1 to a total of 800ng. Cells were harvested after 60 hours by washing once with 1x PBS and extracting luciferase with a luciferase assay kit (Promega) and normalizing against amount of total protein in each extract (measured by Coomassie staining compared to a bovine serum

albumin standard curve). Transfections in primary cells were performed identically, except that twice the amount of cells was used for each reaction.

Lentiviral infections
Measurements were conducted in U2OS cells stably transfected with a circadian *Bmal1-luciferase* reporter, and then infected with Open Biosystems RNAi lentivectors (pGIPZ), as described previously (Maier et al. 2009).

Measurement of circadian bioluminescence in cultured cells
After transfection or infection as described above, circadian rhythms in cell populations were synchronized with dexamethasone, and then measured for 3-5 days via real-time luminometry in normal culture medium lacking phenol red but supplemented with 0.2mM luciferin and 25mM HEPES, as described previously (e.g. Nagoshi et al. 2004). Data were analyzed using the Lumicyle Analysis program (Actimetrics).

cDNA production and quantitative real-time PCR
RNA was extracted as described in Xie and Rothblum 1991. 500ng of total RNA was transcribed to cDNA with SuperScript II (Invitrogen) using random hexamer primers according to manufacturer's instructions. For quantitative real-time PCR 20ng of cDNA was used and single transcript levels of genes were detected by Taqman probes used with the Taqman PCR mix protocol (Roche) using the AB7900 thermocycler. Primers used for detection of NOPS genes are listed below. Primers for detection of circadian genes can be found in Preitner et al. 2002.

Gene	Orientation	Sequence (5' - 3')
NONO	sense	TGC GCT TCG CCT GTC A
NONO	antisense	GCA GTT CGT TCG ACA GTA CTG
NONO	probe	FAM-AGT GCA CCC TTA CAG TCC GCA ACC TT-TAMRA
PSPC1	sense	GAA CTA TAC CTG GCC CAC CAA T
PSPC1	antisense	ACT GCG CC ATTA TCT GGT ATC A
PSPC1	probe	FAM-ATA TTT GCA GCT CCT TCT GGT CCC ATG -TAMRA
SFPQ	sense	TTT GAA AGA TGC AGT GAA GGT GTT
SFPQ	antisense	CTG TTC AAG TGG TTC CAC AAT GA
SFPQ	probe	FAM-TCC TAC TGA CAA CGA CTC CTC GCC CA-TAMRA
GAPDH	sense	CAT GGC CTT CCG TGT TCC TA
GAPDH	antisense	CCT GCT TCA CCA CCT TCT TGA
GAPDH	probe	FAM-CCG CCT GGA GAA ACC TGC CAA GTA TG-TAMRA

Protein Extraction and western blotting
For in vitro immunoprecipitation a 10cm culture dish of HEK 293T cells were co-transfected with each 5ug of NONO-myc, SPFQ-myc or PSCP-myc together with 5ug PER1-FLAG or PER2-FLAG, via polyethyleneimine transfection (JetPEI, Polyplus) following manufacturer's instructions. The cells were harvested 24h later by rinsing with PBS and resuspending in a total of 100ul of lysis buffer as described previously for liver nuclei in Lopez-Molina et al. 1997. Extracts were stored in 500ul aliquots in -80°C until usage. Liver nuclei were prepared by sucrose cushion centrifugation as described in Lopez-Molina et al. 1997, then extracted exactly as for cells. Western blotting was performed using standard procedures (*Current Protocols in Molecular Biology*, Wiley). Equal loading and size detection using protein ladder was verified by Ponceau-S staining of membranes prior to probing.

Immunohistochemistry
Immunohistochemistry was performed according to the protocols described at http://www.pharma.uzh.ch/research/neuromorphology/researchareas/neuromorphology/Protocols/protocol_immuno.pdf. Substrates were either brains collected in isopentane at -20°C and

cryostatically sliced, or cells grown on glass coverslips, rinsed with PBS, and fixed 5' at room temperature in PBS/4%paraformaldehyde.

Antibodies
Polyclonal antibodies against NONO, SFPQ, PSPC1, and PER2 were produced from rabbits by Charles River Laboratories using bacterially-overexpressed proteins. Antibody from each serum was immunopurified over a column whose resin consisted of the relevant antigen covalently coupled to Affygel 10 (BioRad). Anti-PSPC1 is described in Fox et al. 2005. For detection in Co-IP experiments primary anti-MYC antibody (Roche, Cat N°11667149001) was diluted at 1:2000, primary anti-FLAG antibody (Sigma, F3167) 1:2000, primary anti-NONO antibody at 1:2000, primary anti-PSPC1 at 1:1000, primary anti-SPFQ antibody at 1:2000, primary anti-PER2 antibody at 1:1000. The probing of the secondary antibody was done at 1:10´000 for IRDye 680 Goat Anti-Mouse IgG (Licor, 926-32220) and 1:10´000 for IRDye 800 Goat Anti-Rabbit IgG (Licor, 926-33210). For immunoprecipitations, primary anti-cMYC antibody was diluted at 1:500, primary anti-FLAG antibody at 1:500, primary anti-NONO antibody for IP at 1:100, primary anti-SFPQ antibody for IP at 1:100, primary anti-PSCP1 antibody for IP at 1:100 and primary anti-PER2 antibody for IP at 1:100.

Immunoprecipitation
Immunoprecipitation was performed using standard procedures with the below mentioned adjustments (*Current Protocols in Molecular Biology*, Wiley). Extracts were pre-cleared by incubation the crude extracts with protein-A beads (Calbiochem, Cat. N° IP06) and 0.1%BSA for 1h at 4°C. 500ug of pre-cleared extract were bound for 2h to antibody with Co-IP buffer. The Antibody-protein complex was then incubated for 1h with protein-A beads. The beads were washed gently with Co-IP buffer (without protease inhibitor mix) and denatured for 15min at 65°C with 2xSDS sample buffer containing beta-mercaptoethanol. Equal amounts of IP reactions were loaded on a 7% (overexpression in cells IP) or 9% (liver nuclei extracts IP) SDS PAGE gel together with 1/10 of the IP amounts of pre-cleared extract as input. The protein gel and blotting was performed as described in the *western blotting and immunohistochemistry* section above.

Chromatin Immunoprecipitation
Chromatin from mouse liver and tissue culture cells was obtained as described previously (Ripperger and Schibler 2006). Equal amounts of precleared chromatin were incubated overnight at 4°C with 1 ul of anti-NONO antibody or anti-PER2 antibody. The capture of the DNA:protein complexes, the washing conditions and the purifiction of the DNA fragments prior to qPCR as well the control antibodies have been described previously (Schmutz et al. 2010). The region-specific primer/probe pairs are listed in Supplementary Methods.

Paraspeckle Quantification
For paraspeckle detection, after immunodetection of PSPC1 as described above, cells were analyzed with a LSM710 Zeiss confocal microscope. Pictures taken were with 40x (NA1.3), and the pinhole was kept at 1AU or 0.8 to 0.9um. Nuclei were manually detected using ImageJ software routines (http://rsbweb.nih.gov/ij/index.html). Speckles were determined by subtracting background nucleoplasmic PSPC1 protein staining, and thereafter counting remaining pixel clusters in nuclei. The total amount of paraspeckles per cell was estimated by counting all pixels brighter than 140 (arbitrary units) with spot sizes between 0.25-10 squaremicrometers. Nuclei smaller than 200 pixels or 100 suqaremicrometers as well as dividing cells were excluded. The averaged number of speckles was normalized to mean area and compared to the control transfected cells (hairpin NEAT-S).

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Author Contributions

E.K. characterized the *Nono*^{gt}, *Sfpq*^{gt} and *PSPC1*^{gt} mouse lines: Running wheel experiments and expression profiles of genes via qRT-PCR and proteins via Westerns in different organs. J.R. conducted the ChIP experiments. C.M. performed Co-Immunopurification and NEAT knockdown experiments. **E.K.** performed knockdown of NOPS/DBHS homologs in U2OS and bioluminescence recordings. B.M. conducted bioluminescence recordings of NOPS/DBHS homologs overexpression in U2OS cells. S.A.B performed the GAL4 tethering assay in 3T3 cells. **E.K.** did the CDART search on NONO homologs. **E.K.** performed circadian clock transcription activation assay in transient transfections of 3T3 cells. Y.K. contributed overexpression vectors for NOPS/DBHS homologs. A.F. contributed antibody for PSPC1. **E.K.** designed figures. **E.K.** and S.A.B. wrote the paper. All authors made comments on the manuscript.

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Chapter 4 – General Discussion

4.1 Circadian clocks in development

The data presented in chapter 3.1 (Kowalska et al. 2010) proves that there is a functional oscillator present as early as the stage of neuronal precursor cells (NPCs), enabling circadian clock-controlled gene regulation. In pluripotent embryonic stem (ES) cells circadian rhythms are absent. In any case, because these cells divide every 30 minutes, a circadian clock would be difficult to maintain. When ES cells were differentiated into neurons these cells exhibited the same rhythms as adult neurons.

4.1.1 The circadian clock is turned on early during development

Despite this early onset observed in chapter 3.1. it remains unclear if a circadian clock is functional at this stage already *in vivo*. The circadian clock output of behavior is seen only several weeks after birth. However, circadian fluctuations in physiological parameters such as heart beat or hormone levels can be detected in human and mice already prenatally, indicating that the clock is functional as a pacemaker (reviewed in Serón-Ferré et al. 2001). The importance that this synchronized output has in a developing organism is unknown, as phenotypic studies with clock mutant mice have shown that early development is not impaired. This observation would imply that a circadian clock is not required during development.

At an adult stage, however, mutant mice for different core clock genes have severe defects, indicating the circadian clock's importance to maintain homeostasis in an adult organism. *Clock* mutant as well as *Bmal1*$^{-/-}$ mice show defects in glucose homeostasis as found in type II diabetes (e.g. hypoglycemic response) (Rudic et al. 2004, Marcheva et al. 2010) and have fertility problems (Miller et al. 2004, Kennaway et al. 2004, Boden and Kennaway 2006). In addition *Bmal1*$^{-/-}$ mice have an early onset of aging (Kondratov et al. 2006). It is certainly possible that similar types of homeostasis in a developing organism are also under control of the circadian clock, much as it is controlled in adult animals. Although the outcome of this control do not result in obvious deformations such as missing limbs, it is plausible that some of the irregularities observed in adult animals could have their origins at an early developmental stage. Further investigation with developmentally timed clock-deficient animals – e.g. mice with clocks defective only during prenatal or postnatal development – could help to resolve this important question.

4.1.2 Circadian clock-independent functions of core clock genes

During early development, it is also possible that the clock is running for other purposes than to synchronize physiology and behavior with the environment. The development of a multicellular organism requires the specific timing of individual transcriptional programs

and localization of transcriptional regulators (Simpson 2002, Porcher et al. 2010). From cellular decisions of differentiation versus proliferation in early embryogenesis (Molchadsky et al. 2010, Mallanna and Rizzino 2010) to specific cell migration of neuronal progenitor cells in the brain (Borello and Pierani 2010), these developmental programs rely on the presence of specific signaling molecules that are expressed in spatial and temporal patterns. These polarities are established through morphogenes and receive their information of cellular timers to convey starting points for developmental programs (reviewed in Edwards 2003).

Different key players of the core oscillator have been shown to have other roles outside the clock. For example, PER3 is linked to delayed sleep phase syndrome (reviewed in Dijk and Archer 2010), and *per2* shows an elevation in sleep deprived wildtype mice (Franken et al. 2007); specific polymorphisms in the *Clock*, *Bmal1* and *Per3* genes are implicated in bipolar disorders (Benedetti et al. 2003, Mansour et al 2006). Whether this is an indirect effect of the circadian clock via output pathways or a pleiotropic effect of circadian genes is still discussed (Rosenwasser 2010). The fact that *Per* and *Tim* are constitutively expressed in follicle cells of *Drosophila* ovaries (Beaver et al. 2003), and non-circadian PER1 and CLOCK are involved in spermatogenesis resulted in the hypothesis that differentiating cells as found in testis and thymus (Alvarez and Sehgal 2005) arrest their circadian clocks (Alvarez et al. 2003).

One hypothesis for clock function independent of time of day would be to reduce genotoxic stress found in adults (Antoch and Kondratov 2010). The need for effective DNA repair, timed differentiation of precursor cells, or metabolic balance might be important and could be controlled by the circadian clock. The temporal segregation of processes as cellular respiration and cell division might assure more effective DNA repair, thereby enabling genome integrity and stability in the developing organism.

Key players of DNA repair, such as p53, are under the transcriptional control of the circadian clock in adult tissues (Collis and Boulton 2007). For p53, it has also been shown that it can suppress or induce differentiation in pluripotent cells, in addition to its classical role as a tumor suppressor gene (reviewed in Molchadsky et al. 2010). Whereas the circadian control of p53 in adult tissue is implicated in cell cycle control, it could be therefore regulating the fate of progenitor cells in developmental pathways, and timed control for p53 function might play an important role in this process.

To better understand the developmental role of these pathways, it will again be necessary to explore circadian clock-mutant animals and cells specifically during development.

4.2 NONO – a connection between the cell cycle and the circadian clock

The work shown in chapter 3.2 (Paper 2) implies NONO as a new player for the interconnection of the two cellular clocks, the circadian clock and the cell cycle. NONO itself is not under the control of the circadian clock: its transcript and protein levels do not cycle across the day (Brown et al. 2005b), thereby excluding the possibility of an indirect effect through circadian output pathways. NONO binds to the *p16-Ink4A* promoter together with PER2 and acts as a transcriptional activator without influencing transcription levels of other known *p16-Ink4A* regulators such as ETS1. $p16^{INK4a}$ is an inhibitor of the two cyclin D-dependent kinases (CDK4, CDK6) and promotes G1 cell cycle arrest (Ohtani et al. 2004). In $Nono^{gt}$ mice the loss of NONO resulted in its absence at the *p16-Ink4A* promoter and caused abrogation of circadian expression of *p16-Ink4A*.

4.2.1 NONO is a new transcriptional regulator of p16-Ink4A

The observation of hyperproliferation and reduced senescence in $Nono^{gt}$-derived adult dermal fibroblasts (ADFs) led to the investigation of different senescence-promoting pathways depicted in the black boxes in Figure 4.1. Among the three pathways were the mitogen-responsive retinoblastoma pathway, the DNA damage pathway and the telomere pathway. In an RT-PCR Array designed to monitor expression levels of genes involved in the cell cycle, multiple deregulated transcripts showed up. They are labeled with red or green arrows depending on the direction of their regulation in Figure 4.1.

Three key players of different senescence pathways were analyzed in NONO-deficient animals (*Tankyrase*, *p53* and *p16-Ink4A*). *Tankyrase* levels were comparable between wildtype and $Nono^{gt}$ derived skin fibroblasts during passaging and ageing. Previous reports linked telomere shortening to abrogation of circadian clock gene expression and senescence (Kunieda et al. 2006, Qu et al. 2008). The question remains whether abnormal circadian rhythms trigger telomere shortening or if they are a consequence of senescence, which itself is triggered by telomere shortening (Harley et al. 1990). Comparing the transcripts found to be deregulated in $Nono^{gt}$ derived dermal fibroblasts in this study (Table S1 in chapter 3.2) to a recent transcription profiling done in human keratinocytes harboring telomere dysfunction that led to senescence (Minty et al. 2008), no overlap was found. Thus, it is possible that some senescence-promoting pathways are not under circadian clock control.

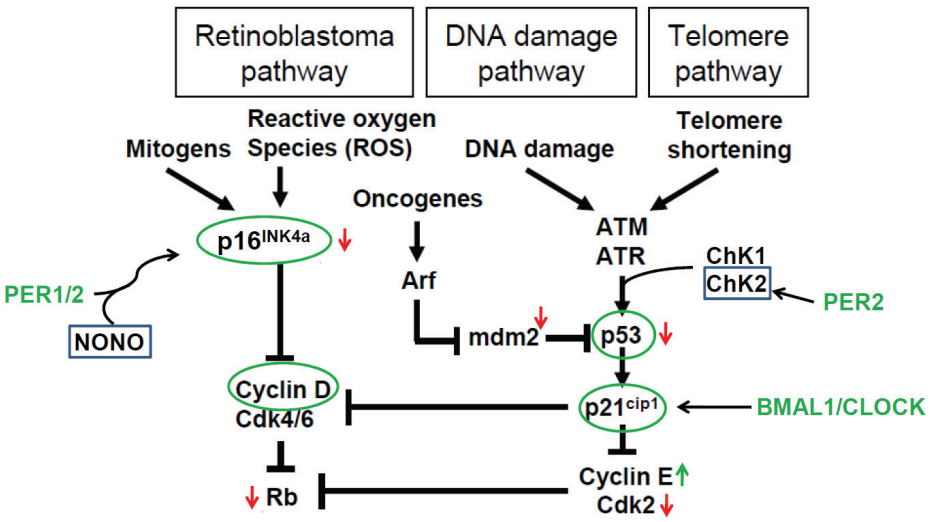

Figure 4.1: Pathways involved in cellular senescence and their regulation by the circadian clock. Three main senescence-activating pathways (black boxes) were checked for transcript level changes using an RT-PCR array (green/red arrows = transcription levels changed in *Nono*^{gt} adult dermal fibroblasts). Green circles indicate proteins transciptionally regulated by the circadian clock. Blue boxes depict binding partners of circadian core clock proteins (in green). (Modified from Chapter 3.2)

p53 and *p16-Ink4A*, both tumor suppressor genes, showed misregulation in *Nono*^{gt} derived adult dermal fibroblasts. Whereas *p53* had half the expression levels of steady wildtype levels throughout the whole time span, *p16-Ink4A* showed an age-dependent increase in wildtype and *Nono*^{gt} derived adult dermal fibroblasts. However, in *Nono*^{gt} fibroblast the overall *p16-Ink4A* levels were twofold lower. Previous studies reported the age-dependent increase of *p16-Ink4A* levels in adult tissue as well as in stem cells and its importance in preventing cancer (Beausejour and Campisi 2006). Similar effects have been reported for a mutant form of *p53* that augments wild-type p53 activity. Heterozygous mice showed early ageing and reduced incidence of spontaneous cancer (Tyner et al. 2002). The possible implications are discussed in the "*NONO - A possible link to cancer*" section below.

The effect of NONO upon *p16-Ink4A* expression levels was dose-dependent indicating a direct role as transcription coregulator. This activation was dependent on the presence of its previously determined binding partners PER1/2 (Brown et al. 2005b): in adult dermal fibroblasts from *Per1*$^{brdm/brdm}$*/Per2*$^{brdm/brdm}$ mutant mice, NONO-mediated activation of *p16-Ink4A* was lost. This may seem counterintuitive, as PER proteins are known to be transcriptional repressors in circadian feedback. Nevertheless, new data starts to question the exclusive negative transcriptional role of PER proteins. Work from Ogawa *et al.* suggests that PER1 and PER2 can potentiate transcriptional activation of BMAL1/CLOCK using a *Per2*::luciferase promoter driving luciferase reporter (Ogawa et al. 2011).

The G1-S phase checkpoint has been previously shown to be under circadian control via checkpoint kinase 2 (Chk2) which interacts in mammals with PER proteins (Gery et al. 2006) and in Neurospora with FRQ (Pregueiro et al. 2006). Chk2 is bound and activated by ATM which then in turn inhibits Cyclin D1, constituting a crucial DNA damage checkpoint. Another circadian control link for Cyclin D1 repression is the the regulation of $p21^{cip1}$ circadian expression by BMAL1 (Gréchez-Cassiau et al. 2008). These links indicate that circadian clock proteins are crucial regulators for the start of DNA synthesis, helping to restrict it to a specific time window. This gating of the cell cycle by NONO was another major finding in the work presented, and shall be discussed in the next section.

4.2.2 Cell cycle gating by the circadian clock requires NONO

The importance of a functional circadian clock for cell cycle control is supported by the FACS data outlined in chapter 3.2. In $Per1^{brdm/brdm}/Per2^{brdm/brdm}$ mutant mice the gating of the S-phase is lost, similarly to $Nono^{gt}$ mice. The link between the circadian clock and the cell cycle was established already previously through circadian transcriptional control of several cell cycle check point proteins like *WEE-1*, *c-MYC* or *Cyclin D1* (reviewed in Reddy et al. 2005). The control by NONO establishes the first direct link of a non-circadian protein that connects both circadian clock and cell cycle.

In unicellular organisms such as the cyanobacterium *S.elongatus* (Yang et al. 2010), the *Euglena gracilis* (unicellular protist) (Carré and Edmunds 1993) or many phytoplankton species (Chisholm and Brand 1981), for example *Gonyaulax polyedra* (Sweeney and Hastings 1958), it has been shown that cell divisions occur in a specific time window every day – i.e. their cell cycle is “gated”. An early on study in the circadian field reported that specifically the S-phase is under circadian control in *Gonyaulax* (Homma and Hastings 1989). Similarly, in the results presented here, NONO regulates *p16-Ink4A*, one of the key players at the G1-S phase transition.

The findings of Dagenais-Bellefeuille *et al.* showed recently that S-Phase in *Gonyaulax* (now known as Lingulodinium) is restricted to the dark period under all different photoperiods tested (Dagenais-Bellefeuille et al. 2008). Thus, not only the length of the cell cycle is fixed, but more broadly the circadian rhythm gates the cell cycle and can be adjusted to different photoperiods. Considering that the circadian clock is an endogenous property, one would expect the cell cycle gating to persist even under constant conditions. In zebrafish cell cycle gating was timed by the circadian clock and was able to persist several cycles after larvae were put to constant conditions (DD) (Dekens et al. 2003).

In mammals different epithelial cells of intestine, tongue, and skin, as well as bone marrow have been shown to possess the same circadian control of S phase gating as unicellular organisms or lower vertebrates (Buchi et al. 1991 García et al. 2001, Clausen

et al. 1979, Smaaland et al. 2002). Even in established cell lines as 3T3NIH that lack systemic signaling Nagoshi *et al.* showed that cells divided during three specific time windows across the day (Emi et al. 2004). Similarly for *Chlamydomonas* it was shown that there are multiple cell divisions occurring during the 6 hours of a "permissive" time window (discussed in Mori et al. 1996).

As 3T3NIH themselves are not photosensitive, they may omit the cell cycle checkpoint governed by ChK2. In addition, Yeom and colleagues showed that cell mitosis in rat-1 fibroblasts was decoupled from the circadian clock (Yeom et al. 2010). They speculate that some immortalized cell lines, though having circadian rhythms, may lose the circadian gating as they hyperproliferate throughout the day. In parallel, using the asebia mouse strain[1] it was demonstrated *in vivo* that hyperproliferating skin fibroblasts lost circadian gating of cell divisions (Brown et al. 1988). This raises the possibility that circadian coregulators of cell cycle checkpoints in these cell lines are not expressed in the right time window and therefore core clock proteins such as PERIODs cannot operate and withhold progress from G1 to S phase to result in hyperproliferation.

It was previously suspected that this control could have an evolutionary reason: to separate light-induced DNA damage from DNA replication (Chen and McKnight 2007). In this context, gating of the cell cycle by the circadian clock would ensure precision and flexibility to predict the light period and to initiate the S phase accordingly (reviewed in Reddy et al. 2005).

The gating of the cell cycle is even observed in unicellular organisms that have cell cycle durations that are distinctly shorter than 24 hour, like cyanobacteria (*S. elongatus*) or green algae (*Chlamydomonas*) (Mori et al. 1996). For *S. elongatus* it has been shown that not the S phase is gated to a specific time window, as DNA content stays constant over the day, but rather cell division (cytokinesis). In circadian clock mutant strains, this "permissive" time window for cell division shifts according to the individual mutant period (Mori et al. 1996). This indicates that, depending on the phyla, different phases of the cell cycle are under circadian control.

The finding of Dong *et al.* that the KaiC protein is regulated through an input pathway protein (CikA) that senses light and metabolism would indicate that cytokinesis is timed according to redox state of the cell. They speculate that the gating of cell division could serve as a synchronizing agent to get equal distributions of circadian clock proteins to daughter cells in a population thereby maintaining the same circadian phase in all individuals (Dong et al. 2010). Also in yeast, there exists oscillations in DNA replication which are antiphase (reductive state) to respirative activities (oxidative state) in order to reduce genotoxic stress (Klevecz et al. 2004).

[1] http://eulep.pdn.cam.ac.uk/~skinbase/mutant_images.php?mutant=Asebia_%28Stearoyl-Coenzyme_A_Desaturase_1%29

4.2.3 Functional circadian clock required for cell cycle coupling through NONO – other physiological functions?

The importance of cell cycle control is crucial for several factors: cells enter cell cycle only when nutrients are available, they proceed only when DNA integrity is assured, and synthesize DNA when genotoxic stress is minimized. This ensures minimal error rate in its genetic material and tissue integrity. The circadian clock might provide this information to the cell, to adjust its division to favorable times of day based upon both metabolic and photoperiodic information. Lacking cell cycle regulation, cells would enter division under unfavorable conditions therefore resulting in premature cell death or accumulation of errors in its genome resulting in cancer precursor cells.

The circadian clock link through NONO might be an additional safety net in the cell cycle pathway. As NONO itself is not regulated in circadian fashion it is possible that it mediates circadian transcriptional control through other binding partners in other cellular pathways. The high expression levels in brain, lung and dermis would indicate regulation of tissue-specific processes. In this context, the impact of NONO depletion on skin fibroblasts will be discussed in the "*Wound healing requires gating of cell cycle by the circadian clock through NONO*".

Higher NONO levels in the brain were confirmed by immunohistochemistry (IHC) in specific regions: the neocortex, the hippocampus and the SCN. In the hippocampus NONO could play a role in motor function and further learning and memory. The latter was recently shown in hamsters to be linked to circadian clocks via the neurotransmitter GABA (Ruby et al. 2008). The circadian master clock in the brain uses GABA to inhibit different parts throughout the day to regulate sleep and wake cycles (Saper et al. 2005). Ruby *et al.* showed that hamsters with a disrupted circadian clock where not able to remember known objects from new ones, a test called novel object recognition task. They reasoned that cyclic GABA release by the SCN enables hippocampal learning at certain times of day. In animals without a circadian clock the chronic inhibition resulted in vanished learning ability. The application of a GABA antagonist inversed this shortcoming in clock mutant mice without altering sleep state (Ruby et al. 2008).

In addition, the dentate gyrus region of the hippocampus is a place of neurogenesis in adult rat, macaque, and humans (Kuhn et al. 1996, Kornack and Rakic 1999, Eriksson et al. 1998) and these neurons display all required parameters to be functional (van Praag et al. 2002). In the past years it has been shown that this new neurons contribute to learning behavior in spatial memory (Nilsson et al. 1999, Dupret et al. 2008, Clelland et al. 2009), but spatial memory does not necessarily need to be associated with neurogenesis (Ambrogini et al. 2004, Gilbert et al. 2005). Therefore the biological role of this phenomenon is still controversial (Leuner et al. 2006).

Whatever is the function, adult neurogenesis takes place only in a very small region of the brain and requires a stringent control of the cell cycle and timed senescence of surplus cells (Lindsey and Tropepe 2006). During neuronal development apoptosis at the G1/S checkpoint disposes of all cells with inappropriate connections or growth. This is executed via upregulation of the CyclinD-CDK4/6 complex activity (Liu and Greene 2001). As NONO is highly expressed in the hippocampus whereas it is absent in most of the rest of the brain, and it controls a senescence checkpoint protein ($p16^{INK4a}$) in the cell cycle, it might be a good candidate gene for exerting this control as increasing $p16^{INK4a}$ in the forebrain decreases neurogenesis during ageing (Molofsky et al. 2006).

To test this idea, a first experiment would be to look in *Nono*^{gt} mice for malformation of the hippocampus due to cell hyperproliferation, and to see if these aged mice accumulate increased numbers of hippocampal cells when NONO is absent. If one could observe hyperproliferation during adulthood, then testing learning and memory would give us an answer if increased cell proliferation in the dentate gyrus improves memory. Independent of the finding it may answer the question of whether decline of cognitive ability with age could result from decreased neurogenesis in adult hippocampus or can be prevented by stimulating it (van Praag et al. 2005).

4.2.4 Wound healing requires gating of cell cycle by the circadian clock through NONO

The gating of the cell cycle by NONO seems to be of crucial importance for tissue integrity in an adult, as wound healing is impaired in *Nono*^{gt} mice. Furthermore, having a non-functional clock, as *Per1*$^{brdm/brdm}$*/Per2*$^{brdm/brdm}$ or *Bmal1*$^{-/-}$ mice, disrupted tissue regeneration in adults after wounding (chapter 3.2).

The observed disruption of timed cell division most likely inhibits proper collagen deposition in *Nono*^{gt} animals. This outcome is counterintuitive, as one would expect better wound healing if hyperproliferation of dermal cells is observed. As the hyperproliferation leads to unorganized tissue layers with reduced collagen levels, the data indicates that the most important impact on impaired wound healing in *Nono*^{gt} animals results from misregulated collagen deposition.

A delay of collagen deposition and slightly reduced levels in collagen I and III were also observed by Ashcroft *et al.* using old animals but still resulted in proper re-epithelialization. In their study however, collagen IV/Laminine were similar from 7 days post-wounding in middle-aged and old animals (Ashcroft et al. 1997). That points to specific and diverged regulation of collagen synthesis pathways, and would point to collagen IV/Laminine as the main players in tissue reconstitution after wounding.

In *Bmal1* mutant mice which have an early aging phenotype (Kondratov et al. 2006), wounds started to close later as well and resulted as well in dramatically reduced collagen deposition and lack of dermal organization. A recent report showed that *Bmal1/Clock* mutant mice develop type II diabetes (Marcheva et al. 2010). Patients with type II diabetes are known to have impaired wound healing which can result in chronic wounds (Greenhalgh 2003). Different factors such as decreased dermal strength of the wound, impaired immune response during inflammation (Greenhalgh 2003) and decreased disposition of collagen contribute to this phenotype and can be partially counteracted by insulin treatment (Schäffer et al. 1997).

Insulin has been shown to accelerate wound healing by increasing collagen deposition while reducing the inflammatory response in wound healing of burns (Madibally et al. 2003). Taken together the data present in chapter 3.2 supports the hypothesis that a certain minimal amount of collagen has to be secreted to result in successful re-epithelialization. Its timing might be controlled by the circadian clock via regulation of insulin levels that were shown to be fluctuating in circadian fashion (Kalsbeek and Strubbe 1998).

4.2.5 NONO - A possible link to cancer

The transcriptional activation of *p16-Ink4A* by NONO would repress entrance to S phase in aging cells and render them quiescent, so called senescent. Cellular senescence as mediated by $p16^{INK4a}$ was found to be an important tumor-suppressor mechanism in ageing cells (Campisi 2005). The resulting hyperproliferation in NONO-depleted skin fibroblasts, enforces the possibility of susceptibility to cancer in $Nono^{gt}$ mice. In addition, Cyclin D1 was found to be overexpressed in many human cancers through the relief of $p16^{INK4a}$ repressor function upon Cyclin D1 (Tetsu and McCormick 1999, Biliran et al. 2005, Knudsen et al. 2006).

One could speculate that other tissues would be prone to proliferate in an uncoordinated fashion, when the cell cycle linker NONO is downregulated or dysfunctional. Developmentally there are no resulting abnormalities in $Nono^{gt}$ mice, but subsequent formation of cancer-like cell populations is not excluded.

Future studies would be needed to show that cell types other than skin that lack NONO will hyperproliferate. As tissue damage occurs every day in the body (e.g. gastro-intestinal tract, skin and mucosa), regeneration would likely require a subset of cells to start proliferating again. These cells presumably require both the coordinating control of the circadian clock to time cell proliferation and secretion of specific factors, as for example collagen for reconstitution of tissue integrity and function. Once the tissue damage is refilled these dividing cells would need to undergo senescence to arrest proliferation. In

Nono^{gt} mice the lack of *p16-Ink4A* seems to prevent this natural process during wound healing and lead to uncontrolled hyperproliferation.

The linkage between wound repair and development of cancer has been reported in several studies (reviewed in Schäfer and Werner 2008, Lin and Karin 2007). A recent study by Wong *et al.* showed, using a mutant mouse model that is susceptible to cancer, that hair follicle stem cells migrate to the site of wounding and express a similar set of oncogenes as found in basal cell carcinomas. After several weeks these cells led to formation of skin tumors at the wounding site (Wong and Reiter 2011).

In addition the lack of proper coupling of the cell cycle to the circadian clock would increase the risk for uncoordinated cell proliferation, if NONO would be a key player in all tissues. In LLC, a tumor-driven cell line, a recent study by Pendergast *et al.* finds the cell cycle to run uncoupled besides an intact circadian clock. These cells were able to divide multiple times throughout the day without circadian gating (Pendergast et al. 2010). This indicates that a functional circadian clock is not sufficient for proper cell cycle coupling, but that there exists a linker.

Nono^{gt} mice have constantly lower levels of p53 as shown in chapter 3.2. This molecular marker has been associated with a higher risk of developing colon cancer and tendency to aneuploidy (Rosman-Urbach et al. 2004). It is possible that this type of cancer results from hyperproliferation of renewing cells which have reduced or diminished NONO levels. This could result at the sites of tissue regeneration in loss of proper G1/S checkpoint control and introduce a continued mass proliferation of cells instead of G0 arrest. In addition, the recent study by Elyada *et al.* showed that p53 downregulation in addition to casein kinase 1α (CK1α) ablation in the intestine, increased invasiveness of non-dividing cells and resulted in tumorigenesis (Elyada et al. 2011). Therefore it would be interesting to monitor NONO levels in cancer cell lines that have downregulated levels of p53, the most frequently mutated in human cancers (Brooks and Gu 2010).

4.2.6 NONO in medical applications

The outcome of the study has possible applications for NONO in medicine. On the one hand, downregulation of NONO at specific times of day where timed collagen secretion is not required would enhance cell proliferation and allow accelerated replenishment of cells after wounding. For example, in patients with large surface destructions (burnings) or old patients that have reduced cell division rates the inhibition of NONO could enhance the natural body response of tissue recovery. On the other hand, for patients with disregulated skin cell proliferation like psoriasis or neurodermitis, increasing NONO levels by topical application could prevent cell hyperproliferation and ensure proper tissue integrity.

4.3 DBHS/ NOPS homologs – a protein family implicated in the circadian clock

Co-regulators or accessory proteins modify the core clock transcripts or proteins in order to turn a simple gene oscillator into an internal timekeeping system with a 24-hour periodicity. In a study conducted previously, NONO was established as a new co-regulator of PER2 (Brown et al. 2005b). Its depletion from the cell resulted in abrogation of rhythms and PER-mediated repression of transcription was more stringent. That led to the model that NONO antagonizes PER-mediated repression (Brown et al. 2005b).

The work conducted in chapter 3.3 (Paper 3) revealed that NONO's presence at the core clock gene promoter *Rev-erbα* is modulating transcription of the respective loci. Furthermore, in *Nono*^{gt} mice the mRNA levels of *Rev-erbα* are misregulated at the specific day times where NONO binding to the promoter peaks. The appearance of RNA-binding proteins in large genomic screens for circadian mutants assumes their implications in proper circadian clockwork but has not been shown directly so far except for the study of NONO (Brown et al. 2005b) and the LARK/RBM4 protein in the *Drosophila* circadian clock (Newby and Jackson 1996, Sofola et al. 2008).

The findings presented in chapter 3.3 now depict that the RNA-binding protein NONO is a co-regulator of transcription. It binds in a PER2 complex to two circadian core clock genes, *Rev-erbα* and *Per2* and the clock-controlled gene *Dbp*. A bioinformatical search revealed that there are two other proteins sharing the unique domain architecture of NONO that combines DNA- with RNA-binding capability, with two RNA recognition motifs (RRM) and an extended DNA-binding domain called NONO/PSP1 (NOPS) domain. This NOPS family consisting in mammals of NONO, SFPQ and PSPC1 are found together or as mixtures in different nuclear complexes or subnuclear domains (reviewed in Shav-Tal and Zipori 2002). There are no homologs of NonA in *Drosophila* explaining probably the severe behavioral phenotype (arrhythmia) found in mutant *NonA* flies (Brown et al. 2005b).

The involvement of two other functional NONO homologs in the circadian clock was shown both *in vitro* and *in vivo* in chapter 3.3. This confirmed that RNA-binding proteins are circadian regulatory elements. However there exist differences among these NOPS homologs concerning mode of action indicating different roles in regulation.

4.3.1 NONO and SFPQ are transcriptional coregulators

Whereas NONO and SFPQ were found at the promoter sites binding in circadian fashion, for PSPC1 no such localization was found. This would be in agreement with previous reports showing that NONO and SFPQ interact directly with each other (Peng et

al. 2002) and both of them have been shown to bind the the activated carboxy-terminal domain (CTD) of RNA polymerase II (Emili et al. 2002, Wu et al. 2006). Furthermore, they play a role in transcriptional control (Mathur et al. 2001, Dong et al. 2007, Basu et al. 1997, Amelio et al. 2007, Urban et al. 2000).

For example, at the thyroid hormone receptor and the progesterone receptor NONO binds together with SFPQ to act as a co-repressor (Dong et al. 2007, Mathur et al. 2001). NONO alone has been shown to activate different promoters (Basu et al. 1997, Amelio et al. 2007) whereas SFPQ on its own is repressing (Urban et al. 2000). In the study of Sewer *et al.* different complexes formed among NONO and SFPQ were investigated based on their transcriptional effects. They found that either, NONO or SFPQ alone was repressing basal transcription at the CYP17 promoter. This promoter can be activated via cAMP when NONO together with the steriodogenic factor (SF-1) are present whereas a complex of all three SF-1/NONO/SFPQ was repressing cAMP-dependent transcription through gene silencing (Sewer et al. 2002).

First indications for this transcriptional gene silencing mechanism were given in a study by Mathur *et al.* showing that SFPQ can interact with the corepressor mSin3A that recruits a HDAC (Mathur et al. 2001). A more recent study from Amelio *et al.*, where NONO binds together with TORC2 to CREB target gene promoters, provided the possible explanation why NONO on its own is activating transcription. In this complex NONO was shown to serve as a connector between the CREB/TORC complex and RNA polymerase II and disruption of this effect by knockdown via RNAi resulted in abrogation of expression of CREB targeted genes (Amelio et al. 2007).

It remains unclear whether NONO and SFPQ can act directly upon a circadian promoter and what their mode of action is. Adding increasing amounts of NONO together with the transactivators BMAL1/CLOCK represses transcription of a circadian promoter (E-box) whereas SFPQ is highly activating transcription. Nevertheless, this is only seen in immortalized 3T3NIH cells: in primary adult dermal fibroblasts (ADFs) both of them are repressing transcription of the same circadian promoter (chapter 3.3). One can speculate that in different cell types there are varying compositions of NOPS homologs at the promoter site and this can result in opposite effects on transcription.

For the *p16Ink4a*, using different deletion promoter constructs fused to luciferase, a specific cis-acting region of 175bp was shown to be important to execute transcriptional activation via NONO. Bioinformatical comparison of this minimal promoter element with the other known NONO-controlled promoters as *Rev-Erbα* or *Dbp* would shed light on common promoter elements. Further comparison with promoters known from the literature to be bound by NONO and SFPQ, would expand the current knowledge of repressive versus activating roles they might play. A new technique, ChIP array (a CHIP on ChIP),

would allow one to screen on a genome-wide level for promoters that are bound by either NONO or SFPQ. The occupied promoters could then be correlated with specific up- or down-regulation of transcripts from exon array data.

4.3.2 Alternative splicing and DBHS/NOPS protein family functions

The different activities of these factors as repressors or activators of transcription could be not only due to complex composition that binds to the promoter but also to different splicing variants of the NOPS homologs. Using the alternative splicing database (ASD[2]) there are eight possible splicing variants for *Nono* (3 confirmed), four possible for *Sfpq* (2 confirmed) and seven possible for *Pspc1* (2 confirmed). One can hypothesize that different splicing variant can localize to different subcellular compartments, interact with different binding partners and perhaps even be expressed at different times of day (Nilsen and Graveley 2010, Keren et al. 2010).

In the midge *Chironomus tentans*, there exists the protein Hrp65 that has been shown to interact in a yeast two-hybrid assay with NONO and other members that contain the DBHS motif (Kiesler et al. 2003). It has three different isoforms: one that contain a NLS (Hrp65-1) and two others that do not (Hrp65-2 and Hrp65-3). All three isoforms arise from the same pre-mRNA through alternative splicing. The NLS-containing splice variant is able to homodimerize with its other splicing variants and shuttle to the nucleus (Miralles and Visa 2001).

All of the NOPS family members have at least 4 alternative splicing patterns (ASP), so one might speculate that also their cellular localization might vary therefore upon the isoform. It would be interesting to find out, if there exist individual isoforms of NOPS homologs and if these vary among the day and are able to bind different subsets of known binding partners that could cause changes in subcellular localization as well as function. One can hypothesize that proteins with multiple isoforms would be produced depending on time of day and their actual function they are supposed to fulfill.

4.3.3 Splicing and post-transcriptional modifications in the circadian clockwork

The proline-glutamine rich splicing factor (SFPQ) has been shown previously to be essential for splicing (Patton et al. 1993). The recent finding that progression of transcript maturation and splicing variant determination is linked to circadian clock work through a methyltransferase (Sanchez et al. 2010) could implicate a transcription-independent role for SFPQ in rhythm generation. The depletion of SFPQ in U2OS resulted in dramatic dampening of oscillations in the first and only cycle. Possible effects would include incorrectly spliced or unspliced and therefore immature transcripts that are dysfunctional.

[2] http://www.ebi.ac.uk/asd/ reviewed in Thanaraj 2004

Designing quantitative RT-PCR primers that monitor exon-intron sites of pre-mRNAs could unravel the presence of immature pre-mRNAs that are now retained in the nucleus.

In *Drosophila* another splicing factor and RNA-binding protein was shown to be essential for the circadian clock as well as development (Markus and Morris 2009). The LARK protein, also known as RBM4, was shown to regulate timing of pupal eclosion. LARK is not expressed in circadian fashion but controls expression of circadian clock output pathways proteins (Newby and Jackson 1996, Huang et al. 2007). RBM4 has different splicing variants that determine cell fate in neuronal stem cell differentiation in developing flies (Brooks et al. 2009).

Considering the embryonic lethal phenotype of homozygous *Sfpq* knockout mice, a developmental role of SFPQ is likely. Interestingly, Lowery and colleagues showed that knocking out *whitesnake,* the zebrafish ortholog of SFPQ, resulted in abnormal embryonic development of neural crest, muscle and heart (Lowery et al. 2007). They found that cell proliferation as well as differentiation was altered in *whitesnake* mutant fish. In mouse *Sfpq* was also found to be highly expressed in brain as shown in chapter 3.3. Investigating how splicing is affected in $Sfpq^{gt/+}$ mice through exon arrays would indicate if there is specific subset of genes that are misregulated.

4.3.4 RNA transport and RNA regulons

To guarantee proper regulation of proteins of the circadian clock or its output pathways, they could get a special escort for transcription, splicing and nuclear shuttling as well as maybe the transport of the mRNA to the cellular compartment. Such RNA regulatory complexes have previously been described as RNA regulons (reviewed in Keene 2007) and mRNA has been shown to travel along the cytoskeleton (reviewed in St Johnston 2005).

Hrp65 would be a protein that has this interesting peculiarity: it binds actin to activate gene transcription via recruitment of histon acetylase transferases (HAT's) and can be found associated to actin of the cytoskeleton in the cytoplasm (Sjölinder et al. 2005, Miralles and Visa 2001). Also NONO is found outside the nucleus in a transport cargo complex that travels along actin in axons of a neuron (Kanai et al. 2004) as well as SFPQ that is involved in translation and shuttles between nucleus and cytoplasm (Sawicka et al. 2008).

The question remains how mRNAs are coupled to cytoskeleton fibers such as actin to be transported along with motor proteins (reviewed in St Johnston 2005) and if maybe the mRNA is loaded inside the nucleus during transcription to monomeric actin (G-actin). Actin as a component of the cytoskeleton was shown to be involved in diverse nuclear functions such as transcription and chromatin remodeling in both prokaryotes and

eukaryotes (Castano et al. 2010, Skarp & Vartiainen 2010). Once outside the nucleus, the G-Actin could polymerize to the existent highways of actin fibers allowing the transcript to travel to its destination. The three DBHS/NOPS family members have been shown to be involved in transcription initiation and transcript maturation, and they shuttle between nucleus and cytoplasm possibly accompanying the protein from its transcription to its translation.

4.3.5 PSPC1 - a nuclear retention machinery for circadian transcripts?

For PSPC1 it has been shown that its localization in the nucleus is mainly restricted to paraspeckles. These are nuclear foci which can retain certain mRNA in the nucleus (reviewed in Fox and Lamond 2010). The *in vitro* data shows clear effects on the circadian oscillator when PSPC1 is overexpressed. The question remains if this results from a direct function of PSPC1 in the circadian clock or by modulating subcellular presence of the other two NOPS homologs and/or complex formations among all three homologs. There is a report which demonstrates that also PSPC1 is a potent activator of transcription in sertoli cells of the testis (Kuwahara et al. 2006). The data presented in chapter 3.3 would not imply an effect of PSPC1 on transcription of the tested circadian promoter (Ebox), and neither does it bind to the promoter of circadian clock gene in a ChIP assay. It could be possible that it serves as a platform to enhance performance or presence of the other two homologs that show involvement in transcription. One can exclude with surety the involvement of paraspeckles in control of circadian rhythms. Neither knockdown nor overexpression resulted in differences of transcription levels of the circadian promoter (Ebox) or period length in cells (chapter 3.3).

4.4 Overall clonclusions and future perspectives

The work of this thesis has enhanced our understanding of co-regulatory mechanisms within the circadian clock and has numerous implications for physiology and development.

The importance of NONO and SFPQ as new transcriptional coregulators for circadian gene expression was demonstrated. Their respective activating or repressive functions depend most probably on cell type and individual promoter "environment". In the future, specific studies of promoter architecture and transcription factor timing at promoters regulated by these proteins would enhance our understanding of their dual transcriptional functions, and provide details of the mechanisms that this thesis began to explore. For PSPC1, transcription-independent roles might possibly involve modifications of RNA shuttling and nuclear retention in collaboration with one or both of the DBHS/NOPS homologs.

Since the NOPS family members are DNA-binding proteins, understanding the role of the RNA-binding domain and its possible involvement in transcription or as a loading platform during transcript maturation could be new aspects of mRNA biogenesis. In addition, finding which RNAs are bound by these new transcriptional coregulators would help to determine if only specific mRNA subsets are controlled in circadian fashion or if they are part of rather general RNA regulatory complexes (RNA regulons).

Furthermore, the circadian control of *p16-Ink4A* and the cell cycle coupling through NONO at the G1/S transition could explain how cancer prevention and pre-mature ageing are kept in balance. As $p16^{INK4a}$ is a senescence-promoting factor during ageing, its loss and the resulting hyperproliferation and impaired wound healing in $Nono^{gt}$ mice could have similar roles in other tissues of the body. Future experiments will show if the homologs of NONO also play a role in ageing and tumorigenesis. Different double knockouts of homologs or even a triple knockout would reveal overlapping actions as well as protein specific functions to better understand their mechanisms of action.

The demonstration that the circadian clock starts ticking early in precursor cells might also imply developmental roles for the DBHS/NOPS proteins. For SFPQ possible implications in development would highlight the importance of RNA-regulated control pathways during embryogenesis and specifically during brain development.

Chapter 5 – Bibliography

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Chapter 6 – APPENDICES

6.1 Acknowledgments

During the past four years I learned what I want and what I do not need. Going through this PhD was hard work and scientific pleasure in one. Many thanks to the people of the Institute of Pharmacology and Toxicology as well as the laboratory members of the Brown group for help and support. More than a “Thank you!!!”, I owe to all the people listed below here, but I think they know that.

Starting as the first PhD student in your lab Steven was not always simple. But I learned how to be patient (even if I still do not excel at it…) when equipment wasn’t ready and how real biochemistry is better than any commercial kit. I thank you for listening and trying to understand my point of view in scientific discussions as well as personal issues. You took my experimental suggestions as serious as the ones coming from a postdoc and I always had the freedom to follow up the things that interested me. Thank you for your trust in me.

As life at the bench is sometimes clouded by not working experiments and personal dilemmas, my deepest thanks go to Ruth Keist, the postdoc I did not have in the first years. Without you Ruth, there would be no data about differentiated embryonic stem cells or hepatocytes that survived the enzymatic digestion. What a lot of hours searching the internet cannot replace, is the knowledge you accumulated during your years of research. And it is more than kind of you, to share your knowledge so eagerly with others. Besides that, you became a true friend to me. Your presence did make more than one of my days.

Sometimes the best friend is the one that went through similar experiences, in that respect Marta Grabiec you were a real revelation. I hardly know any person I connected so quickly to and can finish her sentences or read her look as naturally as yours. You helped me through the rough times and laughed with me through the good ones. I know you only shortly, but I hope that our pathways will cross many more times in the future – and that out of site does not mean out of mind.

I am more than grateful for the patience of my family and friends during the past four years. Dear Mom, you lived through all the ups and downs and always encouraged me to stand up and finish what I started with dedication. Dear Johann and Jadwiga, your quest for a fulfilled work life and self-realization make me always draw my “chapeau”! Dear Franziska, I know you since kindergarten and you know me as good as my family, thanks for all the late night phone calls and support in the past years. Dear Prayanka, we met by chance at a tram stop after your PhD interview. I consider myself very lucky to know you. Your positive approach to problems and your vivid character were good antidotes for my natural critical thinking. And you Brigita, ¡que chica loca!, enligthened with your sarcasm the worst hour and shared with me your fears and dreams. Thank you all, for never having said a bad word, when I turned down an appointment or was too late for one because of my science, you folks always understood what this PhD meant to me, thanks for that!

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During my maturation as a scientist these past years, many excellent mentors have encouraged me on the way and unraveled mysteries worthwhile to spend night and day awake to be solved. They tickled my thoughts and engaged in hours of passionate talk. As young as a college student Dr. Matter and Mr. Bläuenstein, you had me inspired in every hour of teaching you gave. Continuing my studies, the lectures of Prof. Zelewsky, Prof. Meyer, Prof. Boller, Prof. Meins and Prof. Albrecht became true insights in their field of expertise and were shared with the same passion with us students as at their bench. They always fortified my interest and desire to become a “real” scientist.

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Dear Urs (Prof. Albrecht), you became later on a member on my PhD Thesis Committee. Without you and Renée (Prof. Schroeder) these meetings could have easily turned into yearly requisites. But your vivid discussions and scientifically committed nature always made them a date to look forward to.

Many thanks also go to Jürgen (Dr. Ripperger) for the excellent collaborations and lively discussions, and to Robert (Dr. Dallmann) for the last months of discussing and reviewing my work and viewing it in another context. Your points of view were always an impulse to new thoughts and experiments.

6.2 Curriculum Vitae

PERSONAL

Name:	Elzbieta Kowalska
Marital status:	Single
Date of birth:	8th July 1981 in Swidnica, Poland
Nationality:	Polish / Swiss

EDUCATION

November 2006 – April 2011	**PhD Studies** *Focus:* Chronobiology / Neuroscience Institute of Pharmacology and Toxicology at University of Zurich, Switzerland
March 2007 – April 2011	**PhD Program**, Neuroscience Centre Zurich and ETH Zurich
November 2004 – Mai 2006	**Master of Science in Plant Science** Master Thesis title: *Molecular Study of Arabidopsis Genes required for RNA silencing* Friedrich Miescher Institute (FMI) and University of Basel, Switzerland
October 2003 – October 2004	**Bachelor of Science in Biology**, **Major in Integrative Biology** *Focus:* Biochemistry, Plant Biology, Microbiology & Immunology, Ecology University of Basel, Switzerland
September 2001 – September 2003	**1st and 2nd Vordiplom in Biology** University of Fribourg, Switzerland
1996 - 2001	**Matur Typus C** (Mathematical and physical Sciences), Deutsches Gymnasium Biel

LABORATORY EXPERIENCE

November 2006 – April 2011	***PhD studies*****,** Institute of Pharmacology and Toxicology at University of Zurich, Switzerland Laboratory of Prof. S.A. Brown Supervision: Prof. S.A. Brown *Focus:* NONO and its pleiotropic functions
April 2006 – September 2006	***Trainee****,* Friedrich Miescher Institute - FMI, Basel, Switzerland Laboratory of Dr. R. Ciosk *Focus*: Implications of RNA helicases in germ line development of *C.elegans*
February 2005 – March 2006	***Master Thesis Student****,* Friedrich Miescher Institute (FMI) Basel, Switzerland Laboratory of Prof. F. Meins, Jr. Supervision: Prof. F. Meins and Dr. F. Vazquez *Focus:* Mutants enhanced in PostTranscriptional Gene Silencing (PTGS) and expression study of AGO1 and its miRNA regulation

COCURRICULAR ACTIVITIES

May 2008 / April 2009	**"Tage der Genforschung"** Person in support
March 2008	**Brain Fair** Person in support
March 2008 / 2009 / 2010 / 2011	**Assistant** at Bio Modul 420 / University of Zurich
January 2007 – July 2008	**Students Representative** at the Insitute for Pharmacology and Toxicology
October 2005	**Assistant** at Block course in Plant Biology / University of Basel
October 2004	**Assistant** at Block course in Plant Biology / University of Basel
April 2002 – October 2003	**Vice-president** of the Biology Students Association Fribourg

SPECIAL SKILLS

Languages:	*German*	Mother tongue, high school qualifications (Final mark 6, best mark)
	Swiss German	Mother tongue
	Polish	Mother tongue
	English	CAE (12/2005)
	French	DELF 1er et 2eme degrée (12/2005)
	Spanish	TELC A2
Computer:	BioEdit, Vector NTI, Adobe Photoshop CS, LateX Microsoft: Windows XP, Word, Excel, Powerpoint	

6.3 Publications and Talks

Publications:

1. **Kowalska E**, Brügger P, Högger DC, Ripperger JA, Buch T, Birchler T, Müller A, Kramer A, Albrecht U, Contaldo C, and Brown SA.
   NONO couples the circadian clock to the cell cycle. (Revision submitted)
2. **Kowalska E**, Ripperger JA, Muheim C, Maier B, Kurihara Y, Fox A, Kramer A, and Brown SA. 2012. Distinct roles of DBHS family members in the circadian transcriptional feedback loop. *Molecular and Cellular Biology* [Epub ahead of print]
3. Brown SA, **Kowalska E,** Dallmann R. 2012. (Re)inventing the circadian feedback loop. *Developmental Cell* 22(3):477-87 (Review article)
4. **Kowalska E***, Moriggi E*, Bauer C, Dibner C, and Brown SA. 2010. The circadian clock starts ticking at a developmentally early stage. *Journal of Biological Rhythms* **25**: 442-449. (*equal contributing authors)
5. **Kowalska E**, and Brown SA. 2007. Peripheral clocks: keeping up with the master clock. *Cold Spring Harbor Symposia on Quantitative Biology* **72**: 301-305. (Review article)

Talks:

1. **E. Kowalska**, P. Brügger, J. Ripperger, A. Müller, A. Kramer, and S.A. Brown. (July 10th, 2009)
   Characterization of NONO – a Protein with pleiotropic Functions.
   Selected "Short Talk" from poster abstracts. Presented at the Pharmacology Poster Day, University of Zurich, Zürich, CH.
2. **E. Kowalska**, P. Brügger, J. Ripperger, A. Müller, A. Kramer, and S.A. Brown. (July 20th, 2009)
   Characterization of NONO – a new Player in Circadian Clockwork and Sencescence.
   Selected "Hot topic talk" from poster abstracts. Presented at the Gordon Research Conference (GRC) on Chronobiology, Salve Regina University, Newport, USA.
3. **E. Kowalska**, P. Brügger, J. Ripperger, A. Müller, A. Kramer, and S.A. Brown. (September 11th, 2009)
   Characterization of NONO – a new Player in Circadian Clockwork and Sencescence.

Selected “Student Talk” from poster abstracts. Presented at the 6th Horizons in Molecular Biology Symposium, University of Göttingen, Göttingen, DE.

4. **E. Kowalska**, P. Brügger, D. Högger, J. Ripperger, T. Buch, A. Müller, A. Kramer, C. Contaldo, and S.A. Brown. (June 10th, 2010)
   The NONO protein couples senescence, cell cycle, and circadian pathways to regulate wound healing.
   Selected “Short Talk” from poster abstracts. Presented at the Pharmacology Poster Day, University of Zurich, Zürich, CH.